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(54) Title: HIV-TREATMENT METHOD WITH LOW-TOXICITY AMPHOTERICIN B

(57) Abstract

A method of inhibiting HIV infection in peripheral blood macrophages, as evidenced by an inhibition of HIV p24 antigen expression in the infected cells. The method includes exposing the infected macrophages to a composition containing particles of amphotericin B:cholesteryl sulfate, molar ratio 1:0.5 to 1:4, at a concentration of at least about 0.01 μM amphotericin B.

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HIV-TREATMENT METHOD WITH LOW-TOXICITY AMPHOTERICIN B

1. Field of the Invention

The present invention relates to a method of inhibiting HIV infection in HIV-infected peripheral blood cells.

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3. Background of the Invention

Amphotericin B (AMB) is an effective antifungal agent, which at present is the drug of choice for the treatment of a variety of life-threatening systemic fungal infections (1). The drug is presently available for human use as a lyophilized p wder

consisting of a mixture of AMB and deoxycholate known under the trade name "Fungizone." The AMB is known to enter fungal cells and bind strongly to ergosterol, a major sterol component of fungal cell membranes. Ergosterol-bound AMB forms pores in the fungal cell membranes which leads to lysis and cell death. The AMB also has a strong binding affinity for cholesterol, a sterol present in most mammalian cell membranes, and is therefore capable disrupting and damaging host cells.

When AMB is administered in free form, i.e., as a reconstituted AMB/deoxycholate complex, severe side effects are observed. Acute side effects include fever, chills and pain at the site of injection. Dose limiting adverse effects are severe damage to the kidney and anemia caused by hemolysis of red blood cells.

Several studies have shown that AMB toxicity can be reduced by administering the drug in a liposome- bound form (Ref. 2-12). Typically, the LD_{50} of AMB increases from about 2-3 mg/kg body weight for the free drug up to about 8-15 mg/kg when the drug is administered in liposomal form. One limitation of liposomal formulations, however, is the apparent size instability of Amphotericin B/liposomal particles observed when stored in an aqueous medium. Typically, AMB-containing liposomes which have an initial size distribution between about 100-300 nm will spontaneously form aggregates or large liposomal structures of up to several microns on long-term storage in an aqueous medium. Liposomes with sizes greater than about 1-2 microns are generally more toxic than smaller liposomes when administered parenterally, i.e., into the bloodstream.

The toxicity of large liposomes in the bloodstream is related in part to liposome blockage of the alveolar capillaries or capillaries in a peripheral circulation. There are also indications that relatively large liposomes are more toxic to the liver, presumably due to liposome accumulation in reticuloendothelial cells. Co-owned U.S. Patent 4,766,096 for "Stabilized Liposome/Amphotericin composition and Method," discloses a novel method of preparing and storing AMB liposomes which largely overcomes the size-growth problem mentioned above.

An AMB composition formed by complexing AMB with a polyethyleneglycol (PEG) derivative of cholesterol has also been proposed (PCT application US84/00855). This formulation increased the LD_{50} of AMB to 10.0 mg/kg in mice, from 3.8 mg/kg for Fungizone, and was also less cytotoxic in cell culture. It is not known how and whether AMB complexing to PEG-cholesterol affects therapeutic efficacy against fungal infection in vivo, nor whether the complex can be stored in a size-stable form.

More recently, an AMB/cholesteryl sulfate composition sulfate containing a drug:lipid mole ratio of between about 1:1 and 1:4 has been disclosed (U.S. Patent No. 4,822,777). The toxicity of the composition, as measured by LD_{50} in model animals, is substantially lower than that of other reported AMB/lipid compositions, as seen from toxicity studies described below. The composition is therapeutically effective against a number of fungal infections, also as d scribed below. Further, th composition is relatively size stable in

solution, and the particle sizes in the composition are favorable for parenteral drug administration.

It has now been found, in accordance with the present invention, that the low-toxicity AMB/cholesteryl sulfate composition previously reported is effective to inhibit replication of human immunodeficiency virus (HIV) in infected peripheral blood cells, such as monocyte-derived macrophages. HIV is the etiological agent associated with the syndrome of diseases known as acquired immune deficiency (AIDS) and related disorders (25). The host cell range for HIV includes, CD4 T-lymphocyte cells, cells of the mononuclear phagocytic lineage (including monocytes-macrophage), tissue macrophage (27), Langerhans cells, and dendritic reticulum cells (28) of the lymph nodes. Monocyte-macrophage cells are likely a major reservoir of HIV *in vivo*. Further, the monocyte-macrophage cells, either alone or through their interactions with T-cells, may contribute to the development and pathogenesis of HIV related diseases (29).

4. Summary of the Invention

In one aspect, the invention includes a method of inhibiting HIV infection in peripheral blood macrophages, as evidenced by an inhibition of HIV p24 antigen expression in the infected cells. The method includes exposing the infected macrophages to a composition containing particles of AMB:cholesteryl sulfate, molar ratio 1:0.5 to 1:4, at a concentration of at least about 0.01 μM AMB. In a preferred method, the composition contains AMB:cholesteryl sulfate at a molar ratio of 1:1 to 1:2, and particle sizes between about 40-150 nm.

The method may be used for treating a human subject infected with HIV, where the amount of composition administered preferably contains about 0.25-3.0 mg AMB/kg human subject. The treatment method may be carried out by repeated dosing with the composition, until there is produced a measurable improvement in at least one of the indications of HIV infection:

(a) a decrease in HIV antigen levels associated with HIV-infected cells;

(b) a decrease in HIV antigen levels in the bloodstream (antigenemia);

(c) a decrease in the titer of HIV particles in the bloodstream (viremia);

(c) a decrease in the level of reverse-transcriptase activity associated with HIV-infected cells.

(d) a decrease in the rate of HIV-induced destruction of CD4 positive T helper lymphocytes; or

(e) an increase in the absolute number of CD4 positive T helper lymphocytes in the peripheral circulation.

In another aspect, the invention includes a novel method for producing the AMB/cholesteryl sulfate composition, by solvent-injection. In this method, AMB and cholesteryl sulfate are dissolved in DMSO, and injected into an aqueous phase buffer. By selectively controlling injection temperature, aqueous-phase mixing, injection and post-injection incubation times, particles of desired selected sizes, e.g., approximately 100 nm, can be produced.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of Drawings

Figure 1 is a flow chart showing steps in the process for the commercial-scale production of a 1:1 AMB/cholesteryl sulfate composition;

Figure 2 shows the effect of temperature on AMB/cholesterol-sulfate particle sizes in a solvent injection method;

Figure 3 shows the effect of mixing Reynold's number on AMB/cholesterol-sulfate particle sizes in the solvent injection method;

Figure 4 shows the effect of injection time and post-injection incubation time on AMB/cholesterol-sulfate particle sizes in the solvent injection method;

Figure 5 shows electron microscopic images (x 60,000) of negatively stained colloidal dispersions of AMB/cholesteryl sulfate, where the inset shows particle size distribution of the sample;

Figure 6 are graphs showing the inhibition of p24 production in HIV infected macr phage cells treated with AZT;

Figure 7 is a graph showing the inhibition of HIV p24 antigen expression in macrophage infected macrophages following treatment with AMB/cholesteryl sulfate colloidal dispersion; and

Figure 8 is a graph showing the effect of AMB/cholesteryl sulfate dispersion treatment on the viability of the treated macrophages

Detailed Description of the Invention

I. Preparing AMB/Cholesteryl sulfate Particles

This section describes two preferred methods for preparing an AMB/cholesteryl sulfate particle composition for use in the present invention.

A. Thin-Film Hydration Method

In this preparation method, AMB and cholesteryl sulfate are combined in dry form or in solution at a selected mole ratio of amphotericin B:cholesteryl sulfate of about 1:0.5 to 1:4. A solution of AMB/cholesterol derivative in DMSO (dimethylsulfoxide), ethanol, methanol or a combination of these solvents is dried to a thin film by removing the solvent.

The film can be formed from a solvent solution containing a micronized suspension of bulking agent particles (such as lactose), yielding dried particles of the agent coated with the lipid mixture. Solvent removal is by vacuum evaporation or under a stream of inert gas, e.g., nitrogen. The dried lipid film may be stored under an inert gas, preferably at 4°C or less.

An aqueous particle suspension is f rmed by addition of an aque us medium to the dried lipid mixture. The medium used in Example 1, containing

10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4 is suitable. The amount of medium added is sufficient to produce a final AMB concentration of preferably between about 25-100 μmole/ml.

Initially, the lipid material is suspended in the aqueous medium crudely by mechanical agitation until the lipid clumps are released into the aqueous medium to form a slurry-like mixture. This material is then dispersed to a fine particle sizes by sonication, homogenization, French press or other suitable high-energy processing methods. The homogenization process is carried out until a desired particle size, preferably between 40-120 nm is achieved. The suspension may be warmed during dispersion, and should be maintained under an inert atmosphere. In the method described in Example 1, the suspension is sonicated at 4°C to virtual optical clarity. Final particle sizes were between 100-200 nm. Formulations containing less than about 1 mole cholesteryl sulfate per mole AMB do not sonicate to optical clarity, indicating that optimal dispersion requires at least a stoichiometric amount of cholesterol derivative. However, compositions containing a mole ratio of AMB:cholesteryl sulfate of as low as 1:0.5 have been found to have reduced toxicity and desired size characteristics.

The particle suspension may be treated, such as with any molecular sieve chromatography, dialysis or diafiltration, to remove traces of free AMB. The dialysis conditions noted in Example 1 are suitable. The dialyzed material may be lyophilized, for storage, and reconstituted in a suitable injection medium prior to use as a parenteral injectable, as described bel w. The final concentration of AMB in the dispersed particle suspension can be determined

by diluting an aliquot of the suspension in methanol and measuring AMB spectrophotometrically at 406 nm. Typical AMB concentrations at various stages of the preparation of the dispersion are given in Table 1 in Example 1 below.

The dried particle formulation can be prepared either by lyophilization or spray drying. In the former method, the small particle suspension is frozen and lyophilized at a shelf temperature of preferably 2°C or less, as described in Example 1. The effect of lyophilizing on particle size is seen in Table 2 in Example 2, for each of four formulations having AMB:cholesteryl sulfate formulations having molar ratios between 1:1 and 1:4. In each case, mean particle sizes increased from about 100-200 nm before lyophilization, to between 200-300 after lyophilization and rehydration with water. The stability of the particles, pre and post lyophilization is considered below.

For spray drying, the particle suspension is dried in a conventional apparatus in which the particles to be dried are sprayed in aerosolized suspension form into a stream of heated air or inert gas, and the aerosolized droplets are dried in the gas stream as they are carried toward a plate collector where the dried liposomes are collected. An exemplary spray dry apparatus is a Buchi 190 Mini Spray Dryer.

The drying temperature is at least about 37oC, and preferably between about 40-50°C. The temperature of the collection chamber is generally lower than that of the heated air, and typically about 37°C. The dried particles are collected and stored in dehydrated form, under an inert atmosphere.

B. Solvent-Injection Method

A preferred method for the large-scale production of the AMB/cholesteryl sulfate composition for use in the invention is by solvent injection. The preparation will be described with reference to Figure 1, which shows a flow diagram of the steps in the method. Initially, AMB and cholesteryl sulfate, at a selected molar ratio between 1:0.5 and 1:4 (e.g., 1:1), are dissolved in suitable solvent, such as DMSO (dimethyl sulfoxide), or DMSO containing ethanol and/or methanol. The solution may be prepared by heating, e.g., at 50-55° for 0.5-1.5 hours. After solubilization, the mixture is preferably filtered through a 1.2 μ filter to remove particulate material.

The solution of AMB and cholesteryl sulfate is then injected under selected solvent-injection conditions into a suitable aqueous medium. Among the important parameters which have been found to affect final size distribution of the AMB/cholesteryl sulfate particles, in accordance with the invention, are: (a) solvent-injection temperature; (b) aqueous-phase mixing during solvent injection; (c) injection time; and (d) post-injection incubation time. Studies conducted in support of the method indicate that particle size in the final dispersion is relatively unaffected by changes in AMB concentrations, in the range 25-30 mg/ml, and changes in final DMSO concentrations, at DMSO concentrations between about 5-10% by weight. It was also shown that particle size is relatively unaffected by the degree of turbulent flow as it injected fr m a solvent tube into the aqueous medium, and size of injection tube n zzle.

The effect of injection temperature (the temperature of both the injected and aqueous phases) on particle is shown in Figure 2, as a function of injection time. As seen, final particle size was directly related to injection temperature, with the smallest sizes (open circles) being achieved at the highest injection temperatures. Thus, in accordance with one aspect of the preparation method of the invention, it has been discovered that AMB/cholesteryl sulfate particles in the molar range specified above can be prepared in selected size ranges, such as about 40-150 nm, by adjusting the temperature of the injected and aqueous phases during solvent injection.

The mixing within the solvent-receiving vessel can be characterized by a Reynold's number, which a dimensionless number indicating degree of turbulent flow. As seen in Figure 3, higher Reynold's numbers lead to smaller particle sizes.

The total injection time, i.e., the time required for complete solvent injection into the aqueous phase, has a significant effect on particle size. Figure 4 shows plots of AMB/cholesteryl sulfate particle size as a function of injection time. The particle size growth observed can be divided into two phases. The first occurs during actual solvent injection, and shows a linear increase in particle size with time. As seen in the figure, particle size generally increases with increasing injection time, over the 9-495 second times examined.

The second phase of particle growth after injection is completed and shows a quadratic behavi r in particle size increase with time. The rate f particle size growth as a function of time is

slower during this second phase than during the first. The data for Figure 4 was taken at an injection temperature of between 50-55°C. To arrest the size growth of particles during the second phase, the dispersion is cooled to below 30°C. Thus, to achieve a selected particle size, both injection time and post-injection incubation time can be selectively varied.

After solvent injection and incubation is complete, the dispersion is concentrated about 4 fold, then treated by diafiltration to remove solvent, e.g., DMSO, as indicated in the flow diagram in Figure 1. This process results in the spontaneous formation of disc-shaped AMB/cholesteryl sulfate particles having the selected sizes determined by the above-discussed solvent-injection conditions. Figure 5 shows electron microscopic images (x 60,000) of negatively stained colloidal dispersions of AMB/cholesteryl sulfate formed in accordance with the present solvent-injection method. As seen, the particles are disc-shaped, and in this particular composition, have particle sizes between about 80-120nm.

The dispersion formed above is further concentrated by ultrafiltration and the solvent removed by dialysis (diafiltration). The volume is adjusted by the addition of buffer to a AMB concentration of 5 mg/ml and the suspension filtered through a 0.45 micron prefilter. The suspension is further filtered through a 0.22 micron filter and subsequently sterilized by passage through a second sterile 0.22 micron filter.

For us in commercial distribution, the sterile suspension is filled into vials (21.5 ml p r vial) and lyophiliz d under aseptic conditi ns. The

lyophilized preparation is stable in excess of two years when vials are stored at room temperature or below. Prior to administration, the suspension is reconstituted by the addition of sterile water.

II. Physical Characteristics of AMB/Cholesteryl sulfate Composition

This section examines the size stability of AMB:cholesteryl sulfate particle suspensions formed by the two methods described above.

A. Particle-Size Stability

In the first study, reported in Example 2, AMB:cholesteryl sulfate particles having molar ratios of AMB:cholesteryl sulfate of 1:1, 1:2, 1:3, and 1:4 were prepared by thin-film hydration (Example 1) and immediately after dialysis were stored for periods of up to 8 days at 4°C. The results are shown in Table 3. The 1:1 formulation was substantially stable over the 8-day test period, whereas the other formulations showed progressively greater size increases with increasing mole ratios of cholesteryl sulfate.

The size stability of the four formulations after lyophilization and rehydration was determined according to procedures described in Example 1. Size stability data for the eight day test is shown in Table 2 in Example 2. Interestingly, there was little difference in size stability among the four formulations, and for each formulation, mean particle size increased at most about 2 fold over the eight day test period. The combined results from Tables 2 and 3 demonstrate that (a) lyophilized AMB/cholester 1 particles can be reconstituted with little increase in mean size and size distribution

and (b) the particles in the reconstituted suspension are relatively stable on storage in solution over a several-day period.

The effect of physiological-strength saline and plasma on the size characteristics of the particles was also examined, as reported in Example 2. In a first study, the four post-dialysis AMB/cholesterol derivative formulations from above were diluted in 0.9% saline, and the particle sizes examined immediately thereafter. As shown in the top row in Table 4, all of the particles showed a large size increase, although the 1:1 formulation was less aggregated. A similar study on post-lyophilization particles was also carried out, with the results shown in the top row of Table 5 in Example 2. A comparison of the data in Tables 4 and 5 shows that the 1:1 formulation is substantially more size stable in saline after lyophilization than post-dialysis. The other three formulations, having greater cholesteryl sulfate mole ratios, showed large size increases in saline both pre and post lyophilization.

A second study was designed to examine AMB/cholesteryl sulfate particle size in blood plasma, and the effect of subsequent dilution of the plasma medium with suspending buffer. Initially, each of the four samples (both pre and post lyophilization) were diluted 1:1 with human plasma, then diluted after a few minutes with suspending buffer containing 10% lactose. Size measurements were made immediately after dilution, and again 20 minutes later. The results are shown in the bottom two rows f Tables 4 and 5 in Example 2. Summarizing the data, plasma caused a size increase in all of the formulations. Smallest size increases were seen in

the 1:1 formulation, where particle sizes were less than 1 micron (i.e., 1000 nm). The size increase produced on contact with plasma was at least partially reversible for all formulations except the 1:4 formulation, as evidenced by the significant reduction in particle size after 20 minutes incubation in dilute form in suspension medium. There was little difference in the size behavior of particles in pre- and post-lyophilization formulations.

The data above demonstrate that the AMB:cholesteryl sulfate formulation can be stored in dried form long term, without significant increase in size, on rehydration, or significant change in size stability in plasma. One significant advantage of the dried particles which was observed was substantially greater size stability on storage in buffer. Within the range of AMB: cholesteryl sulfate mole ratios which was examined, the 1:1 formulation, gave greatest size stability and smallest mean particle sizes under the various conditions examined.

B. Stability of Particle Composition formed by Solvent Injection

The long-term stability of the AMB;cholesteryl sulfate formulation formed, as above, by solvent injection was examined for both prelyophilized and lyophilized products. Based on the results of several ongoing stability studies, the prelyophilization liquid is stable at 8°C for at least 12 months and at 30°C for 4 months. The lyophilized form of AMB/cholesteryl sulfate dispersion is stable at 8°C and 30°C for at least 12 months and 50°C for at least 6 months. Rec nstituted AMB/cholesteryl sulfate dispersion is stable at 8°C or at ambient temperature, exposed to room light, for at least 7

days, and the reconstituted product is compatible with the stopper. Detailed stability data are summarized in Example 6.

C. Nature of AMB:Cholesteryl sulfate Particles

It has been previously reported that cholesteryl sulfate is capable of forming lipid vesicles or liposomes on extended (several hour) sonication (13). It was therefore of interest to determine whether the AMB cholesterol derivatives particles of the present invention are liposomal in form. For these studies, originally the 1:4 AMB/cholesteryl sulfate formulation was selected, since a relatively high ratio of cholesteryl sulfate is more likely to form liposomal structures. Subsequently, other derivatives were investigated in ratios from 1:1 to 1:4 AMB/cholesterol derivative.

One characteristic of liposomes is a continuous lipid bilayer capable of encapsulating water-soluble solute molecules. Many water-soluble molecules, such as sugars and other marker solutes, are readily encapsulated in liposomes by preparing (dispersing) the liposomal lipids in an aqueous medium containing the marker solute. Smaller marker molecules, such as sugars, also tends to pass through lipid bilayer membranes slowly, as evidenced by equilibration of the solute between encapsulated and bulk phase aqueous compartments over a several-hour to several-day solute-exchange period.

To test the ability of AMB/cholesterol derivative (1:4) particles to encapsulate sucrose, the particles were prepared by dispersion in a medium containing ^{14}C sucrose. After sonication t ptical clarity, the particles were separated from the suspending medium by molecular sieve chromatography,

using a column sieving material which excludes particles in the size range of the AMB/cholesterol derivative particles. Details of the test are given in Example 3. Briefly, 95% of the AMB was associated with the particles eluted in the void volume, but no detectable peak of radioactivity was associated with the particles.

Based on this study, it appears that the particles do not form encapsulating liposomal structure, or alternatively, that the particles form very leaky structures. The latter explanation is unlikely, since (a) cholesterol tends to decrease permeability in liposomes to small water-soluble permeants, and (b) the pure cholesterol derivative liposomes which have been described in Ref. 13 have very low permeability. Studies on cholesterol hemisuccinate liposomes also show stable encapsulation of a variety of small water-soluble molecules (PCT patent application WO 85/05030).

Another characteristic feature of liposomes is the ability of isotonic liposomes to swell on injection into a hypotonic medium. Here the liposomes are acting as small osmometers in response to solute gradients across the bilayer membranes. Isotonic liposome swelling has been observed in liposome prepared from a variety of cholesterol derivatives, including cholesterol-PEG, cholesteryl sulfate (13), cholesterol phosphate and cholesterol hemisuccinate liposomes (PCT patent application WO 85/05030). Cholesterol-derivative liposomes show the expected increased absorbance when injected into increasingly dilute media, although these liposomes behave less lik ideal osmometers than d liposomes formed from c nventional phosph lipid components.

Each of the above four AMB/cholesteryl sulfate particle compositions from above (1:1, 1:2, 1:3, and 1:4 mole ratios) was prepared in 10% lactose. Both pre- and post- dialysis particles were tested for osmotic swelling in distilled water, comparing particle size immediately after dilution with particle size 20 minutes after dilution. The results are shown in Table 6 in Example 3. No swelling was observed in any of the particle formulations. The test supports the finding from the encapsulation studies above that the AMB/cholesterol derivative particles of the invention do not form closed vesicle structures.

III. Reduced Toxicity of AMB:Cholesteryl sulfate Particles

A. Preclinical Studies

As previously reported (U.S. Patent No. 4,822,777), AMB:cholesteryl sulfate particles have a significantly reduced toxicity, when compared with a commercial AMB composition (Fungizone™) and AMB composition formulated in liposomes or other AMB: lipid formulations.

Example 4 describes a number of toxicity studies carried out on AMB;cholesteryl sulfate compositions formed by thin-film hydration. In a first study, reported in Table 7 of Example 4, the LD_{50} of Fungizone™ was compared with that of a 1:4 AMB/cholesteryl sulfate composition. Based on the data shown in Table 7, the LD_{50} of the free AMB (Fungizone™) composition is between 1-4 mg/kg animal weight. This value is increased to between 15-25 mg/kg in the AMB cholesteryl sulfate c mp sition. The data in Table 8 of Example 4 indicate that the 1:1 AMB:cholesteryl sulfate composition has an LD_{50}

value well about 20 mg/ml, and greater than formulations having ratios between 1:2 and 1:4.

The toxicity (LD_{50}) of 1:1 AMB:cholesteryl sulfate particles was compared with that of AMB compositions containing three other cholesterol esters or ethers: cholesterol phosphate, cholesteryl hemisuccinate, and cholesteryl-polyethyleneglycol (PEG). The formulations were all prepared with 1:1 AMB:cholesterol derivative mole ratios, by solvent injection, as outlined in Example 8. The particle sizes, size distributions, and microscopic appearances of the four AMB particle compositions are given in Table 13 of Example 8.

LD_{50} values of the four compositions, and for Fungizone™ in mice was determined by evaluating lethality at increasing AMB doses. As seen from Table 14 in Example 8, all of the cholesterol derivatives reduced AMB toxicity, i.e., increased the LD_{50} severalfold. However, the AMB:cholesteryl sulfate composition was 2-3 times less toxic than the other AMB cholesterol formulations, as evidenced by its 2-3 times greater LD_{50} value.

As part of the preclinical development of AMB/cholesteryl sulfate dispersion, the toxicological profile and dose-related effects of 1:1 AMB/cholesteryl sulfate dispersion, formed by solvent injection as detailed above and in Example 5, have been evaluated in three animal species. AMB, administered as amphotericin B for injection USP (Fungizone™), was included for comparative purposes in selected AMB/cholesteryl sulfate dispersion toxicology studies. These studies provide data that support administrati n of AMB/cholesteryl sulfate dispersion t humans at safe and pharmacologically effective doses.

Six studies, conducted in compliance with GLP regulations, are complete or in progress that provide the essential data describing the toxicology and safety limits of AMB/cholesteryl sulfate dispersion in animals. In addition, LD_{50} values were determined in five separate acute, single-dose studies in mice, and three separate 14-day repeat dose studies were conducted in rats, each at three different dose levels. These eight additional studies (in mice and rats) were conducted early in the development of AMB/cholesteryl sulfate dispersion and were intended to determine dose ranges from which to design the six GLP toxicology studies. Details of the studies are given in Examples 7A-7C.

Overall, the data show the AMB/cholesteryl sulfate dispersion LD_{50} is seven- to tenfold higher than Fungizone™, and repeated dose levels of AMB/cholesteryl sulfate dispersion which are tolerated at least fivefold higher than repeated Fungizone levels, indicating that AMB/cholesteryl sulfate dispersion is significantly less toxic. Further, it was found that with AMB/cholesteryl sulfate dispersion, adverse effects can be reversed without apparent lasting sequelae, following cessation of drug administration, and that the most severe effects occur at doses far in excess of those required for effective antifungal therapy.

B. Clinical Studies

A Phase I study to evaluate the safety of a single dose of AMB/cholesteryl sulfate dispersion administered to healthy male volunteers was conducted, and is detailed in Example 7D. Table 12 in this example lists the incidence of the most common adverse events by dose level, fr m doses of 0.25 t

1.5 mg/kg body weight. In some cases, the incidence and severity appeared to be dose dependent. Other adverse events reported for the active drug which do not appear in this table were lightheadedness, and lips and tongue tingly and numb. These events were reported in the 0.25 mg/kg level only. In addition, chills and trembling sometimes were reported by those volunteers who had an elevated temperature. All laboratory tests performed were within normal limits and no clinically significant changes occurred.

In patients with life-threatening systemic fungal infections, dosing with Fungizone™ is typically started at 0.1 to 0.25 mg/kg and escalated daily up to 0.5 to 0.75 mg/kg or the maximum tolerated dose. Adverse events are common at clinically relevant doses. Premedication with analgesics, such as meperidine, is typically administered to help manage acute effects such as headache, fever, and chills. For example, a retrospective survey of 115 intensively treated cancer patients receiving 91 treatment courses of Fungizone at 0.6 to 0.7 mg/kg found a high incidence of rigors/chills (90%), fever (23%), increased creatinine (52%), and renal toxicity (51%) (Spitzer, et. al. 1989).

Compared to these results, the incidence of adverse effects observed in the Phase I study with AMB/cholesteryl sulfate dispersion, at doses up to 1.5 mg/kg body weight, is consistent with the increased safety margin with AMB/cholesteryl sulfate dispersion compared to Fungizone™ which has been observed in preclinical studies.

IV. Treatment Method

The ability to effectively treat various disease states with the AMB/cholesteryl sulfate composition depends on a number of pharmacologic factors. The first is drug toxicity, which limits both drug dose and patient acceptance of the treatment. The studies reported above and in Example 7 indicate that the AMB/cholesteryl sulfate is administered at substantially higher doses and/or with reduced side effects, relative to prior-art dosage forms of AMB, e.g., Fungizone™. The reduced toxicity of the drug composition is due in part to the controlled, relatively small sizes of the AMB particles (e.g., less than 100 nm). and to the complexation with cholesteryl sulfate. The greater toxicity seen with other cholesterol derivatives complexed to AMB indicates that cholesteryl sulfate confers unique reduced-toxicity properties to AMB.

A second important factor in drug efficacy is pharmacokinetics and tissue distribution. Studies aimed at these factors are given in Example 9. Briefly summarizing the results of these studies, plasma levels after intravenous injection of Fungizone™ or AMB/cholesteryl sulfate particles were higher for Fungizone in the first 12 hours post injection, but lower in the 12-36 hour after injection. Tissue levels of drug were lower in most organs after exposure to AMB/cholesteryl sulfate dispersion, including the major site of dose-limiting toxicity, the kidneys. AMB levels were notably higher in the liver after AMB/cholesteryl sulfate dispersion administration compared to Fungizone. The lower levels of AMB/cholesteryl sulfate dispersi n observed in the kidneys after exp sure t AMB/cholesteryl sulfat dispersion c rrelate with

decreased nephrotoxicity observed for this formulation when compared to Fungizone. Thus, the AMB/cholesteryl sulfate particles appear to be taken up preferentially in the liver, which can act as a slow release depot of AMB in the bloodstream, both limited organ-specific toxicity, and extending the effective dosing interval.

It is also noted, with respect to clearance of the AMB/cholesteryl sulfate, that the cholesteryl sulfate molecules in the particles are natural cholesterol components found widely in animals. The cholesterol compound has no known toxicity, and is metabolized in the body by cholesterol sulfatase.

A third factor in treatment efficacy is, of course, the ability of the drug complex to inhibit or kill target pathogen in the body. A number of studies, reported below and in Example 11, demonstrate that AMB/cholesteryl sulfate particles have significantly enhanced drug efficacy in treating systemic fungal infection. Additional studies carried out in support of the present invention, demonstrate that the AMB/cholesteryl sulfate composition is effective in inhibiting HIV infection in HIV-infected human peripheral blood macrophages, and is thus useful in a method for treating HIV infection.

A. Anti-Fungal Therapeutic Application

The anti-fungal efficacy of AMB/cholesteryl sulfate dispersion has been compared against Fungizone in mice infected with *Coccidioides immitis*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus*. Details of the treatment methods and results are given in Example 10. Briefly, in mice infected with C. immitis, Fungi-

zone™ was effective in clearing coccidioidomycosis infection at 1.3 mg/kg, and was acutely toxic (50% mortality) at 2 mg/kg. AMB/cholesteryl sulfate was effective in clearing infection entirely at 5 mg/kg and no overt signs of toxicity were seen even at 10 mg/kg.

The efficacy of AMB/cholesteryl sulfate dispersion was compared to Fungizone™ in mice infected with *Cryptococcus neoformans*, also as detailed in Example 11. Both AMB/cholesteryl sulfate dispersion and Fungizone were effective in treating murine systemic cryptococcoses with respect to prolongation of survival and reduction of organ burdens of *C. neoformans*. A dose of 3.2 mg/kg Fungizone was toxic, however, and resulted in death of all treated mice. AMB/cholesteryl sulfate dispersion was effective in prolonging survival and reducing organ burdens at 3.2 and 6.4 mg/kg doses, and toxicity with AMB/cholesteryl sulfate dispersion was not seen until doses were 4-6 fold higher than Fungizone (12.8 and 19.2 mg/kg). Similar results were observed in mice infected with *Candida albicans* and *Aspergillus fumigatus*, also as reported in Example 10.

Thus, AMB/cholesteryl sulfate appears to be at least as effective as Fungizone™ at minimum effective doses, and is much better tolerated at elevated doses.

B. Treatment of HIV Infection

Monocyte-macrophage cells which are infected with Human Immunodeficiency Virus Type I (HIV) are known t contribute to the pathogenesis of the immune deficiency associated with HIV infection (30-32). Monocytes originate in the bone marrow, enter

and circulate in the peripheral bloodstream. Some subtypes of monocytes travel to sites such as the liver, spleen and lungs and take up residence in the blood vessels of these organs. Other monocytes migrate deep into tissues such as skin and lymph nodes. Once having taken up residence in these tissues, monocytes can differentiate into macrophages. Macrophages are phagocytic mononuclear cells which actively remove and digest particulate matter such as cell debris, virus particles, bacteria and immune complexes and are critical in antigen presentation. The phagocytic action of tissue macrophages represents a key element of the host immune defense system. These cells may dysfunction by acting as a target and potential reservoir for HIV *in vivo* (19, 20). Monocytes-macrophage have also been implicated in the spread of HIV into the central nervous system (21). In addition to providing a reservoir of HIV in the body, monocytes-macrophage may also be directly involved in the destruction of T-lymphocytes by cell fusion (19).

Experiments performed in support of the present invention demonstrated that amphotericin B/cholesterol derivative compositions inhibit the replication of HIV in infected macrophages, as evidenced by reduced viral production of p24 antigen in monocyte-macrophage cells infected with HIV (Example 11). Briefly, macrophages derived from peripheral blood monocytes were infected with HIV, and treated in cell culture with AZT (obtained from the Burroughs Welcome Company) or with the above 1:1 AMB/cholesteryl sulfate composition. Dilutions of the two drug compositions were added to th c lls at a final drug c ncentration f 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 μg/ml for AMB or 0.01, 0.1, 1, and 10 μM

AZT, immediately following washing of unbound virus inoculum from the cells. Replication of HIV in control (untreated) cell cultures was followed daily, by assay for p24 HIV antigen using a standard p24 antigen capture assay kit (17,18). In addition to using p24 as an indication of viral expression, RNA levels associated with expression of the virus can also be evaluated by slot-blot analysis (22, 23).

Efficacy of the drug action was determined by comparing the concentrations of HIV p24 in treated versus control wells, at a given multiplicity of infection. The results for AZT and AMB/cholesteryl sulfate dispersion are presented in Figure 6 and 7, respectively.

The data plotted in Figure 6 indicate that AZT has the expected antiviral activity against HIV. At multiplicities of infection of 1:3125 or less, AZT at a concentration of 0.01 μM completely inhibits HIV replication in these macrophages. The magnitude of AZT's anti-HIV activity seen here is consistent with those reported in the literature (33). Notice that at multiplicities of infection above 1:3125, 0.01 μM AZT doses not completely inhibit HIV p24 expression. AZT's anti-HIV activity is clearly dependent not only on drug dose, but also on the multiplicity of infection.

In the case of AMB/cholesteryl sulfate dispersion (Figure 7), HIV replication is inhibited in approximately the same concentration range as AZT. For example, at AMB/cholesteryl sulfate dispersion c ncentrations above 0.01 μM essentially complete inhibition of p24 expression is seen. However, in contrast t AZT, inhibiti n of p24 antig n expression by AMB/cholesteryl sulfate dispersi n is not

dependant on the multiplicity of infection. At multiplicities of infection over a broad range, from 1:5 to 1:15625, 0.01μM AMB/cholesteryl sulfate dispersion is quite effective at inhibiting p24 antigen expression.

This lack of dependence of AMB/cholesteryl sulfate dispersion's anti-HIV activity on the multiplicity of infection indicates an important therapeutic advantage over the nucleoside antiviral agents, exemplified here by AZT, which are less effective at high virus titers. HIV-infected patients with high viral burden, such as those experiencing episodes of elevated HIV viremia associated with the acute phase of HIV disease (that occurs within several weeks following the initial infection, often referred to as the acute retroviral syndrome) and those in the late stages of the disease, may benefit more from therapy with AMB/cholesteryl sulfate dispersion than with nucleosides anti-viral agents.

The same logic would apply to health care providers who are accidently infected with HIV by needle-sticks with contaminated needles or surgeons who are exposed to HIV infected blood. Immediate prophylactic treatment of such patients with nucleoside anti-viral agents has not proven effective in preventing the onset and progression of HIV infection. It is believed that immediately following HIV entry into the bloodstream (whether by accidental needle-stick, sexual transmission or any other mechanism) the virus quickly infects cells expressing CD4 (lymphocytes and monocyte/macrophages) and viral replication proceeds at a rapid rate. Newly form d virus particles leave infected cells, travel through the bloodstream and other fluids and infect

greater and greater numbers of susceptible cells. This cycle continues unabated for several weeks since the body has not had sufficient time to mount an effective immune response (i.e., produce effective concentrations of anti-HIV antibodies, a process that takes several weeks to months). During this period the HIV titer in the bloodstream reaches very high levels and it is likely that many (perhaps the majority) of CD4 positive cells are infected. The viral burden is gradually reduced as the anti-HIV antibody levels increase and HIV disease enters its latent phase that can persist for many years.

Since AMB/cholesteryl sulfate dispersion appears to be effective at inhibiting viral replication at high virus titers, it would be indicated particularly for treating the acute phase of HIV-disease. Moreover, immediate administration of AMB/cholesteryl sulfate dispersion following accidental exposure to HIV infected medical devices or body fluids would limit the fulminant progression of HIV infection characteristic of the acute phase of the disease and lessen the severity of symptoms and delay the progression of the disease. Limiting the opportunity for HIV spread by immediate AMB/cholesteryl sulfate dispersion administration following inoculation would further provide an effective prophylaxis.

Cell viability data presented in Figure 7 indicate that AMB/cholesteryl sulfate dispersion is effective at inhibiting HIV replication at concentrations at least two orders of magnitude lower than the cytotoxic levels of the AMB/cholesteryl sulfate dispersion compositi n. Studies performed to investigat the effect of AMB/cholesteryl sulfate dispersion on m nocyte-macrophage cell growth

demonstrated that no significant inhibition of cell growth occurred in the concentration range of 0-5 μg/ml of AMB/cholesteryl sulfate dispersion. These data suggest that there is a sizable therapeutic window (selectivity) for AMB/cholesteryl sulfate dispersion therapy. Therapeutic levels of the drug can be achieved without causing unacceptable toxicity. Pharmacokinetic studies in a 1:1 AMB/cholesteryl sulfate composition indicate that when the compound is administered at a dose of 1.5 mg/kg composition, the AMB concentration remains above the *in vitro* minimum inactivating concentration (MIC) for at least a month. Thus it is possible to maintain effective blood levels of AMB with a dosing schedule that is reasonable for chronic therapy, as would be required to treat HIV disease.

The AMB/cholesteryl sulfate dispersion composition may be inhibiting HIV production by several mechanisms. Like other membrane-bound viruses, HIV is known to contain high amounts of cholesterol in its envelope. Depletion of cholesterol in the viral envelope is believed to reduce the infectivity of a number of such viruses (34). The AMB/cholesteryl sulfate dispersion composition may be interacting directly with mature HIV virions in the culture medium or virus particles budding from the surface of infected cells as has been proposed for the anti-HIV activity of a lipid emulsion formulation known as AL-721 (35). The AMB from the AMB/cholesteryl sulfate dispersion composition may enter the cholesterol-rich viral envelope and bind with high affinity to cholesterol. The presence of AMB-bound cholesterol in th viral envelope may disable virus particles, rendering them unable to productively fuse (enter and infect) additional cells. Alterna-

tively, AMB may transfer from AMB/cholesteryl sulfate dispersion particles into sites in the surface membranes of infected cells where HIV particles are beginning to form and interfere with virus assembly. Areas of virus assembly in the membranes of infected cells are also believed to be enriched in cholesterol content.

It can be appreciated from the foregoing how the ability of the AMB/cholesteryl sulfate composition to inhibit HIV expression in cells can be applied to treating HIV infection in humans. The ability of the AMB/cholesteryl sulfate composition to inhibit HIV replication, either by direct interaction with HIV virus particles in the bloodstream or by entering HIV infected cells, as evidenced by a substantially complete inhibition of viral antigen expression (Figure 7) in infected cells, would reduce the level of infection by reducing the production of virus particles capable of infecting new cells. Further, since the AMB/cholesteryl sulfate composition is effective to reduce expression of HIV in monocyte-macrophage cells, treatment with the composition may be able to reduce the virus reservoirs maintained in these cells.

There is evidence that HIV-infected macrophage fuse with uninfected CD4 expressing lymphoid cells *in vitro* which would provide an additional mechanism for CD4 lymphocyte depletion *in vivo*. The cell fusion process itself can lead to cell death. Depletion of the T-lymphocytes appears to be an important factor in contributing to the progression of HIV diseas and the accompanying secondary consequences of pportunistic infections and n o-plasms (26). HIV pr duced antigens presented on the macrophage cell surface have been implicated in the

formation of syncytia between macrophage and CD4 T-lymphoid cells (19). Since the AMB/cholesterol-derivative composition has the ability to inhibit and substantially eliminate expression of viral antigens in infected monocyte-macrophage cells a marked reduction in this form of cytopathology associated with AIDS would be expected. Even in those cases where infected macrophages do present local patches of HIV antigens on their surface membranes (at sites of viral assembly), AMB delivered in the AMB/cholesteryl sulfate dispersion composition may effectively inhibit the fusion process (and the resulting pathology associated with the formation of syncytia) by binding the excess cholesterol present at these sites.

The AMB/cholesterol-derivative composition can be administered to human patients by a variety of methods and at therapeutic concentrations, as described above and in the section below. The response of the patient to treatment with the AMB/cholesterol-derivative composition can be monitored by evaluating any one or several of the following indications of HIV infection from blood samples collected during treatment of the patient (the so-called surrogate markers of HIV disease progression):

(a) HIV antigen levels, including p24, associated with HIV-infected cells (e.g., by ELISA (17, 18);

(b) HIV antigen levels in the bloodstream (17, 19); (c) the reverse-transcriptase activity associated with HIV-infected cells (36); or

(d) the lev l of replication of the HIV-I virus as identifi d by RNA transcription levels of the viral genome (e.g., slot-blot hybridization (23)).

(e) the level of infectious virus particles in the plasma or associated with formed elements of blood (viremia);

(f) the CD4 cell count in peripheral blood samples.

Maintenance of an antiviral response can be monitored by comparing the levels of the above indicators determined from blood samples taken from the patient before treatment with the AMB/cholesterol-derivative composition to those during or after treatment.

C. Modes of Administration

The present invention provides a dehydrated AMB/cholesteryl sulfate compositions which, when rehydrated after an extended storage period, forms a suspensions of AMB particles having a selected size range less than about 1 micron and preferably between 40-150 nm, and more preferably between 80-120 nm. Because the particles can be stored in an anhydrous, inert environment (or in a vacuum), toxicity and lipid and drug breakdown problems related to oxidation and mechanical damage at a gas/liquid interface are minimized. For parenteral use, e.g., intravenous administration, the compositions were preferably formed from AMB liposomes having sizes of between about 40-400 nm, such as can be prepared by the methods above. The AMB/lipid compositions were hydrated typically to a selected AMB concentration of about 5 mg/ml and then diluted with 5% dextrose to about .63 mg/ml for infusion, and administered at a concentration of between 0.25 and 5 mg AMB/kg b dy weight, and m re preferably ab ut 1.0-3.0 mg/kg b dy weight.

Where the drug is given intramuscularly, to provide slow drug release from the site of injection, the composition is preferably rehydrated to a more concentrated form, which can be conveniently localized in an injection site.

From the foregoing, it can be appreciated how various objects and features of the invention are met. The invention provides AMB formulations which have substantially reduced toxicity and greater drug efficacy than free AMB or lipid/AMB formulations described in the prior art. The enhanced therapeutic index of the drug, particularly related to reduced toxicity, allows much wider use of the drug, for example, for prophylactic treatment of immuno-- compromised patients, and also provides greater therapeutic efficacy in the treatment of active systemic fungal infections.

The compositions of the current invention are readily prepared, the cholesterol derivatives components in purified form are relatively inexpensive, and, being physiologically acceptable, these compositions are naturally utilized when administered parenterally. The formulations are easily stored in dried form, and, when rehydrated, yield a particle suspensions with selected small sizes.

The following examples illustrate methods of preparing, characterizing, and using the AMB/cholesterol derivative compositions of the invention. The examples are in no way intended to limit the scope of the invention.

Materials

Cholesterol 3-sulphat , sodium salt, was obtained from Sigma Chemical Co., St. Louis, MO. AMB

(AMB), USP grade was obtained from Dumex, Copenhagen, Denmark. Sodium cholesteryl sulfate (SCS) was supplied by Genzyme Corp., Farmingham, MA. Cholesterol hemisuccinate and cholesterol iodide were obtained from Sigma, St. Louis, MO. Sodium cholesterol phosphate and 2-(2-methoxyethoxy) ethyl ether of epicholesterol (cholesterol PEG) were synthesized at LTI. Edetate disodium, dihydrate (EDTA), USP, was purchased from Spectrum Chemical Company, Gardena, CA. Trimethamine (Tris) USP, lactose monohydrate, USP, hydrochloric acid (HCl) and dimethyl sulfoxide (DMSO), chromatographic grade, were all obtained from Mallinckrodt, Inc. St. Louis, MD. "FICOLL-HYPAQUE" was obtained from Pharmacia, Piscataway NJ. "RPMI 1640" cell culture medium was obtained from Gibco BRL, Gaithersburg MD. AZT was from the Burroughs Welcome Company.

Example 1

Preparation of AMB/Cholesteryl sulfate Particles By Thin-Film Hydration

AMB and cholesteryl sulfate (CS) in dry powder form were weighed out and combined to give one of the four AMB:cholesteryl sulfate mole ratios listed in Table 1 below. The amount of AMB and cholesteryl sulfate added was sufficient to produce a final AMB plus cholesteryl sulfate concentration in the particle suspension of about 50 umole/ml.

Dry methanol was added to the AMB/cholesteryl sulfate powder to a final AMB concentration of between 0.2-0.6 mg/ml, and the suspension was stirred until all of the powder was dissolved. Lactose was added to this solution to produce a 10% (w/v) lactose s luti n in the final aqueous product. The solution was dried in vacuo, yielding dried

lactose particles coated with a lipophilic AMB/cholesteryl sulfate film.

A suspending buffer containing 10 mM Tris-HCl, 0.1 mM EDTA pH 7.4, 67 mOsm, was added to the dried mixture in an amount sufficient to produce a final AMB plus cholesteryl sulfate concentration of 50 umole/ml. This suspension was sonicated with a probe sonicator (Ultrasonic Liquid Processor, Model W-800, Heat Ultrasonics, Inc., Farmingdale, NY), until the suspension became optically clear. (This process was facilitated when the suspension was warmed to 45°C in a water bath.) Sonication was performed under nitrogen gas.

The sonicated AMB/cholesteryl sulfate particles were dialyzed to remove traces of free unincorporated AMB, using 6000-8000 molecular weight cut-off dialysis tubing. The material was dialyzed against a buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 10% (w/v) lactose, pH 7.4, 300 mOsm. The clear suspension was dried by rapid freezing in a dry ice/isopropanol mixture and lyophilized overnight at a shelf temperature of -25oC, followed by a further two hours at 25°C (15 SRC-X Lyophilizer; Virtis, Gardiner, NY). Lyophilized samples were reconstituted by addition of an equal volume of water and gentle mixing. Table 1 below shows the AMB concentrations of the four compositions, at various stages of preparation.

Table 1

AMB Concentration (mg/ml)

Molar Ratio	Theoretical	Pre-Dialysis	Post-Dialysis	Post-Lyophilization/Rehydration
1:1	23.10	20.66	24.20	21.51
1:2	15.40	10.80	16.46	15.19
1:3	11.55	11.02	12.19	11.60
1:4	9.24	9.49	10.12	8.79

Example 2

Particle Size Characteristics of AMB/CS Composition

A. Effect of Lyophilization on Particle Size

Particle sizes of the AMB/cholesteryl sulfate composition from Example 1 were determined by dynamic laser-light scattering using a Nicomp Model 200 sizer (Nicomp Instruments Inc., Goleta, CA). Samples prepared as described in Example 1 were typically diluted to 0.3 umole/ml for this measurement using 10 mM Tris/HCl, 0.1 mM EDTA, 10% (w/v) lactose buffer, pH 7.4. The mean particle sizes and standard deviations (S.D.) for the four compositions from Example 1 are given in Table 2 below.

Table 2

Molar Ratio AMB/CS	Particles Immediately Post-Dialysis*	Particles Post-Lyophilization/Rehydration*
1:1	138±54	268±137
1:2	148±66	211±105
1:3	172±89	265±147
1:4	138±61	274±151

*Particle Diameter (mean ± S.D. nm)

B. Effect of Storage in Solution on Particle Size

The four samples from Example 1, each containing an AMB plus cholesteryl sulfate concentration of about 50 umole/ml, were incubated at 4°C for up to eight days. At days 0, 2, 6 and 8, an aliquot of each suspension was withdrawn, diluted to about 0.3 umole/ml, and examined for particle size distributi n, as described in Example 1. The results are shown in Tabl 3 below. It is seen that 1:1 composition is stable to particle size change, whereas the

compositions containing higher molar amounts of cholesteryl sulfate are progressively less stable on storage.

Table 3

Days of Storage — Particle Diameter (mean ± S.D. nm) as a Function of AMB/CS Molar Ratio Post-Dialysis

	1:1	1:2	1:3	1:4
0	138±54	148±66	172±89	138±61
2	193±98	336±194	527±299	334±192
6	161±82	462±265	679±377	823±481
8	179±95	512±296	968±551	1034±587

A similar stability study was performed on the same compositions after lyophilization and reconstitution in distilled water, as in Example 1, with the results given in Table 3.

C. Effect of Saline and Plasma on Particle Size

The four samples from Example 1 were diluted to approximately 0.3 umole/ml with 0.9% (w/v) saline and their sizes measured as in Example 2. The results are shown at the top line in Table 5 below.

The four samples were also diluted 1:1 (v/v) with human plasma and subsequently (within a few minutes of contact with the plasma) diluted with 10 mM Tris/HCl, 0.1 mM EDTA, 10% lactose (w/v) buffer pH 7.4, for sizing. Size measurements, reported in Table 5 below, were made immediately after diluting, and 20 minutes after diluting.

Table 4

Particle Size (mean ± S.D. nm) as a Function of AMB/CS Molar Ratio (Post-Lyophilization/Hydration)

Treatment	1:1	1:2	1:3	1:4
dilute in saline	3328±1990	13209±8288	6789±4276	11072±6618
Mix + plasma + suspending buffer	728±334	1200±628	4017±1951	1942±10433
20 minutes later	534±267	776±378	1147±643	2661±1374

Similar size measurements were made on AMB/cholesteryl sulfate particles after lyophilization and rehydration with distilled water. AMB/cholesteryl sulfate in four molar ratios, as described in Example 1, were diluted in saline, or mixed with plasma, diluted in saline and suspended in buffer and the particle sizes were determined at a time 0 and 20 minutes post-lyophilization and hydration. The results are shown in Table 5 below.

Table 5

Particle Size (mean ± S.D. nm) as a Function of AMB/CS Molar Ratio (Post-Lyophilization/Hydration)

Treatment	1:1	1:2	1:3	1:4
dilute in saline	1738±1007	8405±5549	13158±8625	99813±6265
Mix + plasma, dilute + suspending buffer	955±466	1030±550	1766±976	2467±1233
20 minutes later	534±267	776±378	1147±643	2661±1374

Example 3

Structural Characteristics of AMB/CS Composition

A. Encapsulation

AMB/cholesteryl sulfate particles, 1:4 molar ratio, were prepared as in Example 1, except that the Tris buffer medium used to suspend the dried AMB/cholesteryl sulfate mix contained 1 uCi of 14C-sucrose. The suspension was applied to a Sephadex G50 gel exclusion column equilibrated with 10 mM Tris/HCl, 0.1 mM EDTA, 10% (w/v) lactose buffer, pH 7.4, and the applied material was eluted with the same buffer. The particles were eluted in the void volume, which was monitored by UV absorption at 280 nm. The samples were collected and examined for radioactivity by conventional scintillation counting.

B. Osmotic Swelling

AMB/cholesteryl sulfate formulations containing the four different mole ratios of AMB and cholesteryl sulfate were prepared as in Example 1, (in the usual suspension medium containing 10% lactose). These samples are designated as post-dialysis (P.D.) suspensions in Table 6 below. A portion of each sample (containing 10% lactose) was lyophilized and reconstituted in distilled water, and these samples are designated as lyophilized and reconstituted (L.R.) in the table.

The P.D. and L.R. samples were each diluted to 0.3 umole/ml with distilled water, and the size distribution of the particles immediately after dilution in the hypotonic medium, and 20 minutes after dilution was measured as in Example 1. The results are given in Table 6 below. As seen, there is no appreciable swelling, over a 20 minute incubation period, as evidenced by an increase in mean particle size, in any of the samples examined. Combined with the results presented in Example 2 on the lack f solute encapsulation, these data indicating a lack of osmotic

activity confirm that the 1:1 - 1:4 AMB/Cholesteryl sulfate compositions are not conventional liposomes.

Table 6

Molar Ratio AMB/CS	Sample Post-Dialysis (PD) or Post-Lyophilization/Rehydration (LR)	Time (in minutes)	Particle Size (mean ± S.D. nm)
1:1	PD	0 20	109±42 114±45
1:1	LR	0 20	212±107 212±105
1:2	PD	0 20	126±59 134±64
1:2	LR	0 20	170±81 184±89
1:3	PD	0 20	124±58 131±61
1:3	LR	0 20	205±117 204±107
1:4	PD	0 20	161±86 185±100
1:4	LR	0 20	228±116 236±122

Example 4

Biological Properties of AMB/CS Composition

A. Toxicity (LD_{50}) of the Particle Suspension

Outbred male Swiss/Webster mice were obtained from Simonsen Labs, Inc. The animals weighed approximately 15-45 grams on the day f treatment and were between 4-8 w eks old. The animals were quarantined for at least three days prior to the study, and only mice that remained

healthy during the quarantine period were used. The animals were given food and water ad libitum.

Animal groups were treated with either a Fungizone-free form of AMB obtained from Squibb suspended in sterile saline or 1:4 AMB/cholesteryl sulfate composition prepared as in Example 1. In each case, the AMB concentration was adjusted so that the selected dose of AMB (given in Table 7) could be administered in a final volume of 0.2 ml. The test material was administered by a single intravenous injection via the lateral tail vein. Each dose was administered over about 1.5 minutes.

The animals were observed for signs of toxicity and death at least three times (1, 2, and 4 hour post treatment) on the day of treatment. During the remaining observation period of five days, the animals were examined daily in the morning and afternoon. The test results, expressed as the ratio of number of survivors on day five:total number of animals treated, are given in Table 7.

Table 7

Number of Survivors on Day 5 Post-Injection/Total Animals Injected

	Treatment Animals Total Injected	Survivors/Day 5 Post Injection
FUNGIZONE		
0.5 mg/kg	8	8
1.0 mg/kg	8	8
2.0 mg/kg	8	8
4.0 mg/kg	8	2
6.0 mg/kg	8	0
8.0 mg/kg	4	0
AMB/CS (1:4)		
10 mg/kg	3	3
15 mg/kg	4	3
20 mg/kg	5	2
25 mg/kg	3	1

In a second toxicity test, mice were treated with 20 mg/kg of one of the four AMB cholesteryl sulfate formulations from Example 1, with drug administration and animal monitoring being done as above. The results are presented below in Table 8.

Table 8

Number of Survivors on Day 5 Post-Injection/Total Animals Injected

	Treatment Animals Total Injected	Survivors/Day 5 Post Injection
AMB/CS		
1:1 molar ratio	6	6
1:2 molar ratio	5	0
1:3 molar ratio	5	1
1:4 molar ratio	5	0

B. Efficacy of the AMB/Cholesteryl sulfate Formulation

Crl:CFW (SW) BR mice weighing 20-25 grams were obtained from the Charles River Breeding Laboratories and were given food and water ad libitum. C. albicans strain 30 was grown at 35°C on SDA (Sabourund Dextrose Agar) for 18 hours, and the organism was harvested and diluted with sterile nonpyrogenic saline to yield about 7 x 10?8 colony forming units in a 0.2 ml volume.

Eight-ten animals per group were injected in the tail vein each with 0.2 ml of the above C. albicans mixture. Two days after the fungal injection, the animals were injected with graded doses of Fungizone or AMB cholesteryl sulfate (1:4) prepared as in Example 1. The AMB preparations were adjusted in c ncentration s that each animal r ceived a total volume of 0.1 ml, administered intravenously through the tail vein.

The amount of AMB administered, expressed in terms of mg drug/kg body weight of the animal is given at the left in Table 10. The animals were followed for 25 days postdrug administration. The number of survivors at 25 days per total number of test animals is shown in the Table 11 for the two AMB preparations, and a buffer control.

Table 10

DOSE	FREE AMB FUNGIZONE	AMB/CS 1:4	CONTROL
0.1 mg/kg	-	-	0/11
0.3 mg/kg	2/10	5/10	-
0.6 mg/kg	1/10	9/10	-
0.9 mg/kg	3/10	9/10	-
2.0 mg/kg	-	10/10	-

Example 5

Preparing 1:1 AMB/Cholesteryl sulfate Composition By Solvent Injection

This example describes a method of preparation suitable for large-scale (commercial scale) production of 1:1 AMB/cholesteryl sulfate sodium salt colloidal dispersion by solvent injection.

A total of 300 g AMB and 158 g of CSSS (1:1 molar ratio) were dissolved in 11 Kg DMSO by heating for 0.5 hours at 50-55°C. The solution was then injected, using a gear pump, into a stainless steel jacketed processing tank containing 90 Kg of 5 mM Tris-HCl buffer, and 0.1 mM EDTA adjusted to, pH 7.4. The residual DMSO was removed by diafiltration. The dispersi n was first concentrated d wn to about 1/4 of its volume and then diafiltered against 10 volume exchanges of th above buffer. When diafiltration

had been completed, 9.5% (w/v) lactose was added in the dispersion while stirring. The volume was then adjusted to an AMB concentration of 5 mg/ml and the dispersion was sterile filtered. The resulting product was an isotonic, yellow opalescent suspension free of aggregation as examined by a phase contrast light microscope (Leitz, Dialux 20). Electron microscopic images of negatively stained AMB/cholesteryl sulfate particles indicated the presence of disc like particles, rather than spherical liposomal structures (Figure 1).

For use as an injectable suspension, the above suspension was lyophilized. Reconstitution is in a suitable reconstitution medium, to give a final injectable suspension having the components given in Table 11 below.

Table 11

	Prelyophilization (mg/ml)	Reconstituted solution (mg/ml)
AMB	2.50	5.00
CS (sodium salt)	1.32	2.64
Tromethamine USP	0.30	0.60 (5 mM)
EDTA USP	0.02	0.04 (0.1mM)
Lactose	47.50	95.00
Water for injection	qs to 1.0 ml	qs to 1.0 ml

Example 6

Stability of AMB/Cholesteryl sulfate Dispersion

Stability studies of prelyophilization material from above, and reconstituted composition were conducted. Based on the results of several ongoing stability studies, the prelyophilization liquid is stable at 8°C for at least 12 months and at 30°C for 4 months. The lyophilized form of AMB/cholesteryl sulfate dispersion is stable at 8°C and 30°C for at least 12 months and 50°C f r at least 6 months. Reconstituted AMB/ch lesteryl sulfate dispersion is stable

at 8°C or at ambient temperature, exposed to room light, for at least 7 days, and the reconstituted product is compatible with the stopper. Detailed stability data are summarized below.

A. Stability of Liquid (Prelyophilized) Drug Product

Stability studies of prelyophilization liquid AMB/cholesteryl sulfate dispersion were conducted with amphotericin B at strengths of 2.5 mg/mL and 5.0 mg/mL. Individual 10-cc Type-1 clear glass vials were filled with 5 mL of the prelyophilization liquid, stoppered with siliconized grey butyl lyophilization-type stoppers, sealed with aluminum seals, and placed at various temperatures in an upright position, protected from light. At designated time points, vials were removed and assayed.

For the 2.5 mg/mL prelyophilization liquid, data are available at three storage temperatures (8°C, 30°C, and 50°C) from one batch for up to 12 months. The stability profile was the same as for the 2.5 mg/mL liquid.

B. Storage Conditions for Prelyophilized Composition

Based on stability study results the intermediate, prelyophilization liquid stored at 2°C to 8°C, protected from light, was given a 5 week expiration date. This duration is acceptable for handling and storage of the bulk prelyophilization liquid during manufacturing at the 60-L process scale.

C. Stability of Lyophilized Composition

Stability studies of lyophilized AMB/cholesteryl sulfate dispersion were conducted with amphotericin B strengths at 50 mg and 100 mg per vial. Individual 50-cc Type-I cl ar glass vials, containing th lyophilized AMB/cholesteryl sulfate dispersion, stopper d with silic n-ized grey butyl lyophilizati n stoppers and sealed with

aluminum seals were placed at various temperatures in an upright position and protected from light. At designated time points, vials were removed and reconstituted with sterile water for injection (10 mL for 50 mg/vial and 20 mL for 100 mg/vial) to yield a 5 mg/mL amphotericin B solution. The reconstituted samples were then assayed.

For the 50 mg dosage vials, data are available from two batches at three storage temperatures (8°C, 30°C, and 50°C) for up to 18 months. For the samples stored at 8°C and 30°C, all vials tested were within the product specifications. For the 50°C samples, the amphotericin B level was the only component that did not meet the product specification beyond 6-7 months of storage.

For the 100 mg dosage vials, data are available from one batch at three storage temperatures (8°C, 30°C, and 50°C) and another batch at 30°C for up to 12 months. A third batch was also stored at 30°C and data are available for 6 months. The same stability profile was obtained with amphotericin B strengths at 100 mg and 50 mg. At the two lower storage temperatures (8°C and 30°C), all vials tested were within the product specifications. Samples stored at 50°C were stable for at least 6 months.

D. Storage Conditions for Lyophilized Composition

Based on these updated stability results, the expiration dating of lyophilized AMB/cholesteryl sulfate dispersion stored at controlled room temperature and protected from light has been extended from 6 months to 12 months. The stability of lyophilized AMB/cholesteryl sulfate dispersion used in clinical trials will be continuously monitored to insure its integrity.

E. Compatibility of Reconstituted Composition With a Stopper

To determine compatibility of reconstituted AMB/cholesteryl sulfate dispersion with the stopper, stability studies of the reconstituted vials stored in an inverted position were conducted. Individual 50-cc Type-I clear glass vials, containing 100 mg lyophilized AMB/cholesteryl sulfate dispersion, were stoppered with siliconized butyl stoppers and sealed with aluminum seals. The lyophilized product was reconstituted with 20 ml Sterile Water for Injection using a sterile syringe and needle. For the light stability, the reconstituted vials were inverted and placed at 8°C or at ambient temperature exposed to room light. Assays were performed on day 1 and day 7. For the freeze/thaw stability, the reconstituted vials were inverted and were placed at -7°C for 24 hours and were thawed at 8°C until assays were performed.

All vials tested are within the product specifications, and no trend is apparent. These results indicate that reconstituted AMB/cholesteryl sulfate dispersion is compatible with the lyophilization stopper and is stable at 8°C or at ambient temperature, exposed to room light, for at least 7 days.

F. Recommended Storage Conditions

While the physical and chemical data support a prolonged shelf life of reconstituted AMB/cholesteryl sulfate dispersion, the product is stored in the refrigerator and is given an expiration dating of 7 days because the product is not preserved.

Example 7

Toxicity of AMB/cholesteryl sulfate Composition

A. Toxicity Studies in Mice

The LD_{50} of both AMB/cholesteryl sulfate dispersion and Fungizone was determined in male and female Swiss Webster mice by evaluating lethality at 20, 30, 40, 50, 60, and 70 mg/kg for AMB/cholesteryl sulfate dispersion; and 2, 3, 4, 5, and 7 mg/kg for Fungizone. In males, the intravenous LD_{50} for AMB/cholesteryl sulfate dispersion is 36 mg/kg and for Fungizone, 2.6 mg/kg. In females, the intravenous LD_{50} for AMB/cholesteryl sulfate dispersion is 38 mg/kg and for Fungizone, 2.0 mg/kg. Doses higher than 5 mg/kg Fungizone caused 100% mortality in both male and female mice. In contrast, mortality did not exceed 90% at doses as high as 70 mg/kg AMB/cholesteryl sulfate dispersion.

B. Toxicity Studies in Rats

The rat was used as a test species because of the existing information on amphotericin B effects in this species and its suitability for use in toxicology and pharmacokinetic studies. Compared with humans, rats appear to tolerate similar dose levels of amphotericin B (Parekh et al, 1975). Three toxicity study studies were conducted in rats: 14, 28, and 91 days of consecutive daily dosing.

Two 14-day repeat dose-ranging studies and one 14-day pharmacokinetics and tissue distribution study were performed in Sprague-Dawley rats. These showed that daily intravenous administration of up to 6.0 mg/kg AMB/cholesteryl sulfate dispersion for two weeks was tolerated by rats with no observed morbidity or loss of body weight, whereas all animals that received more than 1 mg/kg Fungizone died or became moribund during these studies.

In an ther study, rats were exp sed t AMB/cholesteryl sulfate dispersion at doses of 1 and 5 mg/kg/day, and Fungizone at a d se of 1 mg/kg/day for 14 days in a compa-

rison study of pharmacokinetics and tissue distribution. Clinical chemistry, hematology, and urinalysis parameters were measured during the two-week exposure and then for an additional two weeks. Changes in urea nitrogen levels and urine osmolality during the study indicated that similar renal effects occurred with 5 mg/kg AMB/cholesteryl sulfate dispersion and 1 mg/kg Fungizone; at 1 mg/kg AMB/cholesteryl sulfate dispersion, urea nitrogen levels were only slightly elevated from controls. These effects correlated with tissue amphotericin B levels in all three groups and were reversed readily in all groups after cessation of dosing. Although this study showed that liver levels of amphotericin B were higher with AMB/cholesteryl sulfate dispersion than Fungizone, no indications of hepatotoxicity were observed even at a dose of 5 mg/kg AMB/cholesteryl sulfate dispersion.

These studies show that rats were able to tolerate AMB/cholesteryl sulfate dispersion at levels at least sixfold higher than Fungizone after two weeks of repeated dosing. Effects of AMB/cholesteryl sulfate dispersion on the kidneys were reversible and no toxicities unique to AMB/cholesteryl sulfate dispersion were observed.

A GLP study of the toxicity of 28 days of consecutive repeat dosing of AMB/cholesteryl sulfate dispersion was conducted in Sprague-Dawley rats. Both sexes were given doses of AMB/cholesteryl sulfate dispersion at 1.0, 2.5, and 5.0 mg/kg/day. As a comparison another group received 1.0 mg/kg of Fungizone. Some animals were necropsied after 28 daily doses; others were observed for an additional 14-day recovery period and then necropsied. Drug-related effects were assessed by recording body weight, food consumption, clinical observations, ophthalmoscopy, clinical pathology (hematology, clinical chemistry, and urinalysis), gross pathology, and micr scopic path logy.

Dose-related elevations of urea nitrogen were observed in animals exposed to both AMB/cholesteryl sulfate dispersion and Fungizone. These changes reversed during the 14-day recovery period. Hyperplasia of the renal and urinary bladder epithelium were observed in animals exposed to AMB/cholesteryl sulfate dispersion as well as those exposed to Fungizone. These hyperplasias did not reverse within the 14 days for either formulation. No toxicities unique to AMB/cholesteryl sulfate dispersion were observed in this study. The frequency and severity of drug-related effects observed after 5.0 mg/kg AMB/cholesteryl sulfate dispersion were less than or equal to those observed after 1.0 mg/kg of Fungizone, indicating a fivefold margin of safety for AMB/cholesteryl sulfate dispersion over Fungizone.

Another GLP toxicity study in rats is nearing completion. In this study rats received 0, 2.5, 5.0, or 7.5 mg/kg for 91 consecutive daily doses of AMB/cholesteryl sulfate dispersion by intravenous bolus injection. Some animals were necropsied at the end of 91 days; the remainder will be necropsied at the completion of a 45-day recovery period. Plasma samples are being collected for drug content analysis after 45 and 90 days of dosing and at the completion of the recovery period.

C. Toxicity Studies in Dogs

Four studies were conducted in beagle dogs. The dog was chosen as the test species, not only because of the large reference database available, but because the dog is known to be very sensitive to toxicities induced by amphotericin B, particularly gastrointestinal effects.

The single dose toxicity of AMB/cholesteryl sulfate dispersion in dogs was determined after intravenous administration as either b lus injection or slow infusion (2-3 hours). Bolus injection was at doses 5, 10, 15, and 25 mg/kg and infusi n at 15 mg/kg. Assessment of the

health of each animal included measurement of body weight, clinical observations, clinical pathology studies (hematology, clinical chemistry, and urinalysis), gross pathology, and microscopic examination of tissues in the event of death.

Some effects were seen at all doses, ranging from minor body weight loss at lower doses to severe distress which required euthanasia within seven hours of dosing with animals receiving 25 mg/kg, and within six days with animals receiving 15 mg/kg by bolus injection. Slowing the rate at which the 15 mg/kg dose was administered by changing from bolus to infusion significantly reduced gastrointestinal effects and increased the survival of the animals.

Clinical signs of toxicity occurred at doses of 10 mg/kg AMB/cholesteryl sulfate dispersion and higher but were absent at 5 mg/kg AMB/cholesteryl sulfate dispersion. These clinical signs were consistent with gastrointestinal irritation. Results of clinical laboratory tests, such as the concentrations of hepatic enzymes in the serum, indicated toxic effects on the liver, and abnormal blood urea nitrogen and creatinine concentrations indicated toxic effects on the kidneys.

Changes in these serum chemistry parameters occurred at all AMB/cholesteryl sulfate dispersion doses, but the effects ranged from mild in the lower dose groups to severe only in the two highest bolus dose groups. Values for serum chemistry parameters increased transiently; however, there was no other indication of liver or kidney dysfunction, and in all other regards, the animals were free from any adverse effects at the bolus dose of 5 mg/kg AMB/cholesteryl sulfate dispersion.

Most importantly, recovery was complete from both renal and hepatic effects 14 days after dosing in all surviving animals. The post-mortem pathology in this

animals euthanized earlier in the study revealed lesions consistent with hemorrhage, principally in the gastrointestinal organs and the lungs. There was an apparent sex difference in the susceptibility of these dogs to some of the adverse effects of AMB/cholesteryl sulfate dispersion when administered at high doses. The females seemed to be more resistant to the effects and had a greater facility to recover from changes in hepatic and renal function.

A repeat dose study was designed to evaluate the acute toxicity of AMB/cholesteryl sulfate dispersion when administered intravenously by bolus injection daily for 14 consecutive days. This study was conducted in male and female beagle dogs at doses of 0.6, 1.2, 2.5, 5, and 10 mg/kg AMB/cholesteryl sulfate dispersion and compared with Fungizone at 0.6 mg/kg. The animals were assessed by clinical observations and measurements which included body weight, hematology, clinical chemistry, and urinalysis, and gross and microscopic pathology in animals that died or were euthanized.

In the animals that received 0.6 and 1.2 mg/kg doses of AMB/cholesteryl sulfate dispersion, no adverse effects were observed or measured. In contrast, animals administered 0.6 mg/kg Fungizone showed severe renal toxicity; at post mortem examination, these animals also showed severe gastric and intestinal hemorrhage.

Dosing with AMB/cholesteryl sulfate dispersion at 2.5 mg/kg and higher resulted in mild body weight depression, increased incidence of clinical signs of toxicity, and changes in clinical chemistry parameters associated with renal and hepatic function. However, the dogs dosed with Fungizone had toxicities and histopathologic lesions which did not become evident in AMB/cholesteryl sulfate dispersi n-dosed animals until d ses of 10 mg/kg were reached. It was therefore concluded that the high st dose level of AMB/cholesteryl sulfate dispersion appropriate for longer

term daily administration in dogs should be not greater than 2.5 mg/kg, because at this dose, the first changes in blood urea nitrogen and serum creatinine concentrations were measured. It was further concluded that when prolonged, consecutive daily dosing is administered, AMB/cholesteryl sulfate dispersion has at least a twofold margin of safety relative to Fungizone.

Beagle dogs were exposed to AMB/cholesteryl sulfate dispersion for 30 consecutive days by bolus intravenous injection. Doses of 0, 0.5 1.0, and 2.0 mg/kg/day were administered. As a comparison, another group of animals received 0.4 mg/kg/day of Fungizone. Necropsies were performed after dogs received 30 doses. This study was an interim report of a 13-week study.

Drug-related effects were assessed by observing body weight, food and water consumption, clinical observations, vital signs, ophthalmoscopy, clinical pathology (hematology, clinical chemistry, and urinalysis), gross pathology and microscopic pathology.

No treatment related effects were observed in dogs exposed to AMB/cholesteryl sulfate dispersion at 0.5 mg/kg for 30 days. Reversible, dose-related nephrotoxicity was observed at higher levels of AMB/cholesteryl sulfate dispersion and after Fungizone (0.4 mg/kg). No significant toxicities unique to AMB/cholesteryl sulfate dispersion were observed in this study. The magnitude of drug-related effects observed after 2.0 mg/kg AMB/cholesteryl sulfate dispersion was similar to that observed after 0.4 mg/kg Fungizone, indicating a fivefold margin of safety for AMB/cholesteryl sulfate dispersion.

In this GLP study, beagle dogs of both sexes were exposed to AMB/cholesteryl sulfate dispersion for 91 consecutive days by bolus injection at doses of 0, 0.5, 1.0, and 2.0 mg/kg/day, with 0.4 mg/kg/day of Fungizone as comparison. Necr psies were performed after 91 days in

four animals per sex, while two others were observed during an additional 56 day recovery period before necropsy.

Drug-related effects were assessed by observations of body weight, food and water consumption, vital signs, clinical observations, ophthalmoscopy, clinical pathology (hematology, clinical chemistry, and urinalysis), gross pathology, and microscopic pathology. Plasma concentrations of amphotericin B were measured at several time points during the study.

All dose levels of AMB/cholesteryl sulfate dispersion and Fungizone were tolerated in this study (dogs received up to 182 mg/kg total cumulative dose of AMB/cholesteryl sulfate dispersion). No significant gross pathologic effects were observed in any group during the 13 weeks of exposure or 8 week recovery, and clinical observations were limited to occasional occurrence of mucoid feces.

Significant, dose-related nephrotoxicity was observed in all groups exposed to AMB/cholesteryl sulfate dispersion or Fungizone. Dose-dependent increases in serum creatinine and urea nitrogen, and decreases in urine osmolality and specific gravity were correlated with microscopic observations of tubular nephrosis and nephrocalcinosis. The severity of these changes was somewhat greater in animals exposed to 0.4 mg/kg Fungizone than in those exposed to 2.0 mg/kg AMB/cholesteryl sulfate dispersion, indicating a safety margin of at least fivefold for AMB/cholesteryl sulfate dispersion over Fungizone. Reversibility of these lesions during the recovery period appeared to be slightly slower in animals exposed to Fungizone.

Severity of lesions observed after 13 weeks of exposure to AMB/cholesteryl sulfate dispersion were not substantially increased from those after 30 days of exposure, and no irreversible drug-related effects were observed.

Minimal to mild, statistically significant alterations in platelet counts, erythrocyte counts, hematocrit, hemoglobin and haptoglobin occurred, mainly in AMB/cholesteryl sulfate dispersion dosed animals. These changes were not associated with any specific lesion, and were considered to be of limited clinical significance. In this study, males appeared to be more sensitive than females to both AMB/cholesteryl sulfate dispersion and Fungizone. Exposure to AMB/cholesteryl sulfate dispersion resulted in reduced incidence of local inflammation at the injection site compared to exposure to Fungizone.

The liver, spleen, lymph node, and bone marrow associated macrophages were examined extensively for evidence of lipid accumulation which might occur with a lipid-based colloidal formulation. Although increased numbers of pigmented macrophages were observed in the livers of all animals exposed to 2.0 mg/kg AMB/cholesteryl sulfate dispersion by week 13, this also occurred in some animals exposed to 0.4 mg/kg Fungizone. This finding reversed during the recovery period and was not judged severe enough to achieve biological significance. No significant lipid accumulation was found to occur during 13 weeks of exposure to AMB/cholesteryl sulfate dispersion, at cumulative doses up to 182 mg/kg.

Analysis of plasma samples after 30, 60, and 90 days of dosing and after 28 days of recovery demonstrated that drug plasma amphotericin B levels in animals dosed with 2.0 mg/kg AMB/cholesteryl sulfate dispersion were identical to those in animals exposed to 0.4 mg/kg of Fungizone, and that plasma levels during the recovery period fell at almost the same rate with both formulations.

This study further supports the finding that dose-related effects produced by AMB/cholesteryl sulfate dispersion are qualitativ ly similar or identical t those observed with Fungizone, but these effects only occur at

doses of AMB/cholesteryl sulfate dispersion at least 5 times greater than Fungizone.

In all of the studies conducted in mice, rats, and beagle dogs, pharmacological effects and toxicities of AMB/cholesteryl sulfate dispersion are qualitatively similar to those seen with other forms of amphotericin B; that is, the effects and toxicities were those commonly seen with the drug, and no new ones were introduced with the AMB/cholesteryl sulfate dispersion formulation. The renal and hepatic changes are well established dose-related effects of amphotericin B as is the hemorrhaging seen in the beagle dog. It is most significant that, with AMB/cholesteryl sulfate dispersion, adverse effects can be reversed without apparent lasting sequelae, following cessation of drug administration, and that the most severe effects occur at doses far in excess of those likely to be required for effective antifungal therapy.

D. Phase I Safety Study in Human Volunteers

A Phase I study to evaluate the safety of a single dose of AMB/cholesteryl sulfate dispersion administered to healthy male volunteers was conducted at the University of Utah, Drug Research Center. Plasma samples were obtained from each subject at the beginning, mid-point, and end of drug infusion and at time points up to 28 days after administration. These samples were analyzed for amphotericin B content by HPLC.

In this study, 23 healthy adult male volunteers were enrolled in a randomized, double-blind, placebo-controlled evaluation of AMB/cholesteryl sulfate dispersion. Safety and tolerance were assessed in six volunteers at each of 4 dose levels. At each dose level, four volunteers received the active drug and two received placebo with the xc ption of the 0.5 mg/kg dose level for which 3 volunteers received active drug and 2 received placebo. The dose levels were

assessed sequentially to allow evaluation of safety before administration of the next higher dose in the next group of volunteers. For ethical reasons, the investigator did not include a comparison with Fungizone.

A test dose of 1 mg of AMB/cholesteryl sulfate dispersion was administered 24 hours before the administration of the study dose to determine if the volunteer had an intolerance to the study drug. Vital signs were taken before infusion and at 1, 2, 4, 6, and 12 hours after infusion. The test dose was well tolerated in all volunteers. Tolerance to the study dose was based on physical examination, vital signs, clinical laboratory tests and adverse events. The study doses tested were 0.25, 0.5, 1.0, and 1.5 mg/kg. Vital signs were taken prior to, at midpoint, at end of infusion, and at 1, 2, 4, 10, 24, and 48 hours after the end of infusion. Adverse reactions were monitored throughout the time the volunteer was in the study center and at return visits for blood sample collection. No premedication was given to any of the volunteers to minimize adverse events.

No unique adverse events were reported by volunteers who received AMB/cholesteryl sulfate dispersion; they were those typically seen with Fungizone.

Seventy-three percent of the volunteers receiving AMB/cholesteryl sulfate dispersion (11 of 15) had one or more adverse events reported. A total of 49 adverse events were reported for the volunteers on drug, with 41 events rated by the principal investigator as mild and 9 reported as moderate. None of the adverse events were rated as severe. Fifty percent of the volunteers receiving placebo (4 of 8) had one or more adverse events, with 10 reported as mild, 1 moderate and 1 severe.

Table 12 lists the incidence of the most common adverse vents by dose level. In some cases, the incidence and severity appeared to be dose dependent. Other adverse events reported for the activ drug which do not appear in

this table were lightheadedness, and lips and tongue tingly and numb. These events were reported in the 0.25 mg/kg level only. In addition, chills and trembling sometimes were reported by those volunteers who had an elevated temperature. All laboratory tests performed were within normal limits and no clinically significant changes occurred.

In patients with life-threatening systemic fungal infections, dosing with Fungizone is typically started at 0.1 to 0.25 mg/kg and escalated daily up to 0.5 to 0.75 mg/kg or the maximum tolerated dose. Adverse events are common at clinically relevant doses. Premedication with analgesics, such as meperidine, is typically administered to help manage acute effects such as headache, fever, and chills. For example, a retrospective survey of 115 intensively treated cancer patients receiving 91 treatment courses of Fungizone at 0.6 to 0.7 mg/kg found a high incidence of rigors/chills (90%), fever (23%), increased creatinine (52%), and renal toxicity (51%) (Spitzer, et. al. 1989). Compared to these results, the incidence of adverse effects observed in the Phase I study with AMB/cholesteryl sulfate dispersion is consistent with the increased safety margin with AMB/cholesteryl sulfate dispersion compared to Fungizone which has been observed in preclinical studies.

Pharmacokinetics of AMB/cholesteryl sulfate dispersion was also determined in the human subjects. Blood samples for analysis of drug serum levels were collected before, at midpoint, and end of infusion; and at 15 and 30 minutes, and 1, 2, 4, 7, 10, 24, and 48 hours after infusion; and then at 7, 14, 21, and 28 days after infusion. A 12-hour urine collection was obtained before administration of the study drug, and two serial total 24-h ur urine collections were obtained after dosing, while the volunt er was in the study center.

The plasma kinetics observed after intravenous infusion of AMB/cholesteryl sulfate dispersion in this study were similar to those previously reported for Fungizone: rapid distribution, large volume of distribution and long elimination half-life. Peak levels of amphotericin B in plasma, observed at the end of the infusion, had mean values of 0.805, 0.843, 2.191, and 2.534 ug/mL after doses of 0.25, 0.5, 1.0, and 1.5 mg/kg, respectively. Rapid distribution after the infusion was followed by a biexponential elimination phase with a terminal half-life of 86 to 243 hours.

AMB was detected in the plasma for 28 days following infusion of 1.0 and 1.5 mg/kg AMB/cholesteryl sulfate dispersion. Total body clearance ranged from 1.5 to 2.0 L/hour (0.018-0.043 L/hr·kg) and the volume of distribution ranged from 250 to 650 liters (3.0-10.0 L/kg).

Plasma concentrations of drug increased with increasing doses of AMB/cholesteryl sulfate dispersion, resulting in a linear increase in area under the curve (AUC) over the range of doses studied. The apparent increases in half-life and volume of distribution with dose may be the result of inadequate characterization of the terminal elimination phase of the lower doses because plasma levels dropped below the level of detection.

Table 12

Incidence of Adverse Events by Treatment/Single-Dose Study in Healthy Male Volunteers

	AMB/CS Dispersion Treatment Group (mg/kg)					
Adverse Event	Total	Placebo	0.25	0.50	1.0	1.5
Backache	6	1	0	0	3	2
Chills/Hot	7	2	0	1	1	3
Headache	8	2	1	1	2	2
Nausea	10	2	2	2	2	2
Vomiting	3	0	0	1	0	2
Pain at infusion site	5	0	0	0	2	3
Other	21	4	4	1	8	4
Total	60	11	7	6	18	18
Number of Volunteers	23	8	4	3	4	4

Example 8

Comparison of Toxicity in AMB/cholesterol Formulations

The solvent injection system described in Example 5 was used to prepare AMB compositions containing 1:1 AMB with cholesterol phosphate, cholesterol hemisuccinate, and cholesterol polyethyleneglycol (PEG), as described below. These three formulations were compared for toxicity (LD_{50} in mice) with the AMB/cholesteryl sulfate composition prepared in Example 5.

A. Preparation Methods

AMB/Cholesterol Phosphate. A total of 100 mg AMB and 53 mg of cholesterol phosphate were dissolved in 12 ml DMSO and 25 ml methanol. The solvent mixture was then injected into 213 ml of buffer. Upon completion of diafiltration

and concentration, it yielded about 20 ml of the colloidal dispersion. The final product was slightly aggregated and required sonication before filtration. The particle size determined by a laser particle sizer was 42 nm. Electron microscopic images of the dispersion also indicated disc shaped particles but size was much smaller than that of AMB/cholesteryl sulfate colloidal dispersion shown in Figure 1.

AMB/Cholesterol Hemisuccinate. A total of 250 mg of AMB and 155 mg of cholesterol hemisuccinate was dissolved in 12 ml DMSO and 6 ml methanol. The mixture was injected into 240 ml buffer. Upon completion of diafiltration and concentration, it yielded about 50 ml AMB/cholesterol hemisuccinate colloidal dispersion. The product was free of aggregation with mean particle size of 48 nm. Smaller disc shape particles were seen by electron microscopy.

AMB/Cholesterol-PEG. A total of 125 mg of AMB and 66 mg of cholesterol PEG was dissolved in 7.6 ml DMSO and 3.8 ml of methanol. The mixture was injected into 140 ml buffer. Upon completion of diafiltration and concentration, it yielded about 25 ml AMB/cholesterol PEG colloidal dispersion. The product was heavily aggregated and was unable to be filtered through a 0.22 micron membrane. The mean size was 132 nm with much greater size distribution (34% versus 22-26% for the other cholesterol derivative formulations).

B. Particle Characteristics

The three AMB formulations were examined for particle sizes, morphological appearance, and cholesterol content, as described above, with the results shown in Table 13.

Table 13

Particle Size, Size Distribution and Microscopic Appearance of AMB Colloidal Dispersion formed with Various Types of Cholesterol Derivatives

Type	Size[1] (nm)	Size Distribution (%)	Microscopic Appearance[2]
Cholesteryl Sulfate	98	22	No aggregation Free of crystals
Cholesteryl Phosphate	42	24	No aggregation Free of crystals
Cholesteryl Hemisuccinate	48	26	No aggregation Free of crystals
Cholesteryl PEG[3]	132	34	Very aggregated Free of crystals

1. Particle size was determined by a Nicomp Model 200 laser particle sizer.

2. Microscopic examination was performed by a Leitz, DIALUX 20 phase contrast light microscope.

3. Sample for cholesterol PEG was unfiltered due to heavy aggregation.

In the cholesterol phosphate, and hemisuccinate formulations, no aggregation and no crystals were observed, and particle sizes were 42 and 48, respectively. The cholesterol PEG formulation was aggregated but was free of crystals.

C. Toxicity

The acute toxicity of three AMB/cholesterol derivatives formulations, plus AMB/cholesteryl sulfate, was measured according to the same protocol and compared to the acute toxicity of Fungizone.

In two separate studies the result of which are combined in Table 14, the LD_{50} (and 95% confidence interval) of AMB/cholesteryl sulfate dispersion was found to be 78.0 (48 +/- 10) mg/kg and the LD50 of Fungizone was 3.5 (2.5 +/- 0.1) mg/kg. The LD50 values for complexes with cholesterol phosphate, cholesterol hemisuccinate and cholesterol polyethylene glycol were 16.2 (11-22) mg/kg, 24.7 (18 - 35) mg/kg and 23.9 (14-41) mg/kg respectively. All three cholesterol derivatives reduced the acute toxicity of AMB with respect to Fungizone, but none was less toxic than AMB colloidal dispersion (AMB/cholesteryl sulfate dispersion).

Table 14

	LD_{50}	95% Conf. Int.
Fungizone*	3.3 3.7	2.4 - 5.1 2.6 - 5.1
AMB/CD* Chol. Sulfate	68.0 87.7	38.2 - 110 59.4 - 128
AMB/Chol Phosphate	16.2	11 - 22
AMB/Chol Hemisuccinate	24.7	18 - 35
AMB/Chol PEG	23.9	14 - 41

Example 9

Pharmacokinetic Distribution of AMB/CS Composition

Pharmacokinetics and tissue distribution of amphotericin B after intravenous administration of AMB/cholesteryl sulfate dispersion have been studied in the rat and dog.

A. Pharmacokinetics and Tissue Distribution Studies in Rats

Two pharmacokinetic studies have been conducted in rats: a single dose study and a 14-day repeat dose study.

In the single dose study, Sprague-Dawley rats were given AMB/cholesteryl sulfate dispersion by intravenous bolus injection at 1.0 or 5.0 mg/kg. As a comparison, Fungizone was administered at 1.0 mg/kg. Plasma levels were significantly lower in animals exposed to AMB/cholesteryl sulfate dispersion compared to Fungizone during the first 12 hours. The terminal elimination half-life was longer after AMB/cholesteryl sulfate dispersion; thus, at later timepoints (over 36 hours) drug plasma levels were higher in AMB/cholesteryl sulfate dispersion-dosed animals. Tissue levels of drug were lower in most organs after exposure to AMB/cholesteryl sulfate dispersion, including the major site of dose-limiting toxicity, the kidneys. AMB levels were notably higher in the liver after AMB/cholesteryl sulfate dispersion administration compared to Fungizone.

Increasing the dose of AMB/cholesteryl sulfate dispersion fivefold, from 1.0 to 5.0 mg/kg, did not result in significantly higher plasma or kidney levels than observed after exposure to 1.0 mg/kg Fungizone. The less than proportional increase in plasma levels observed indicates dose-dependence of AMB/cholesteryl sulfate dispersion metabolism in this species.

After Fungizone administration, amphotericin B was found to be widely distributed, mainly in the liver, lungs, spleen, and kidneys. In contrast, nearly 100% of the administered dose was recovered from the liver 30 minutes after AMB/cholesteryl sulfate dispersion was administered.

The lower levels of AMB/cholesteryl sulfate disp rsi n observed in the kidneys after exposure to AMB/cholesteryl

sulfate dispersion correlate with decreased nephrotoxicity observed for this formulation when compared to Fungizone.

In a second study in rats, amphotericin B levels during a 14-day repeat dose study were significantly lower in plasma and most tissues after administration of AMB/cholesteryl sulfate dispersion compared to an equivalent dose of Fungizone (1 mg/kg). When the dose of AMB/cholesteryl sulfate dispersion was increased to 5 mg/kg, plasma levels and most tissue levels still did not significantly exceed those produced by 1 mg/kg of Fungizone. Drug at either dose did not accumulate in plasma. Drug plasma levels reached steady state during the dosing period. With AMB/cholesteryl sulfate dispersion, drug levels in plasma and tissue showed a somewhat longer terminal elimination half-life during the two-week washout period, compared to Fungizone.

Concentrations of amphotericin B were significantly lower in the kidneys with AMB/cholesteryl sulfate dispersion compared to Fungizone (both at 1 mg/kg); the lower kidney levels from AMB/cholesteryl sulfate dispersion were accompanied by reduced nephrotoxicity.

Concentrations of amphotericin B were significantly higher in the liver with AMB/cholesteryl sulfate dispersion compared to Fungizone (both at 1 mg/kg). While amphotericin B levels in the liver were higher after AMB/cholesteryl sulfate dispersion, increased hepatotoxicity was not observed.

B. Plasma Pharmacokinetics Studies in Dogs

In a 14-day repeat-dose study in dogs, administration of AMB/cholesteryl sulfate dispersion resulted in lower plasma levels of amphotericin B than Fungizone at equivalent doses (0.6 mg/kg). Plasma levels from AMB/cholesteryl sulfate dispersion reached st ady state during the 14-day

dosing period, whereas drug levels continued to rise in dogs receiving Fungizone during this period.

Tissue levels of amphotericin B in the kidneys and gut, key sites of amphotericin B toxicity in the dog, were significantly lower with AMB/cholesteryl sulfate dispersion than with Fungizone at equivalent dose levels. As with the rat, lower drug levels in the kidney from AMB/cholesteryl sulfate dispersion were accompanied by reduced nephrotoxicity.

With repeat dosing in dogs, higher amphotericin B levels were produced in the liver from AMB/cholesteryl sulfate dispersion compared to Fungizone at equivalent doses. However, these higher levels were tolerated without increased hepatotoxicity. When AMB/cholesteryl sulfate dispersion was administered at 5 mg/kg, dogs tolerated 7-9 times more amphotericin B in the liver before exhibiting hepatotoxicity equivalent to that seen with Fungizone at 0.6 mg/kg. Thus, AMB/cholesteryl sulfate dispersion is less hepatotoxic than Fungizone.

Significantly less amphotericin B was recovered in the urine and feces after AMB/cholesteryl sulfate dispersion administration than after Fungizone. However, the fact that urinary and biliary clearance rates were the same with both indicates that renal and hepatic excretion mechanisms were unaffected.

Example 10

Anti-Fungal Therapeutic Application

The anti-fungal efficacy of AMB/cholesteryl sulfate dispersion has been studied in mice infected with *Coccidioides immitis*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus*.

A. *Coccidioides immitis*

Three studies were done to determine the maximum tolerated dose and therapeutic range of AMB/cholesteryl sulfate dispersion and Fungizone against *Coccidioides immitis* in female CD-1 mice. After an initial study to determine dose tolerance, animals were infected with virulent *C. immitis* and treated on days 3, 5, 7, 10, 12, and 14 post-infection with buffer controls, AMB/cholesteryl sulfate dispersion (0.22, 0.66, 1.3, 2.5, 5.0, 7.5, or 10 mg/kg), or Fungizone (0.22, 0.66, 1.3, or 2.0 mg/kg). Animals were monitored for 28 days post treatment, at which time surviving animals were killed. Lungs, livers, and spleens were removed and the burden of remaining infection determined.

AMB/cholesteryl sulfate dispersion was effective in treating murine systemic coccidioidomycosis. Further, the formulation was well tolerated, and the mice did not show overt signs of toxicity. AMB/cholesteryl sulfate dispersion completely cleared the infection from all animals treated i.v. with 5.0, 7.5, and 10 mg/kg. The lower doses of AMB/cholesteryl sulfate dispersion (0.22, 0.66, 1.3, and 2.5 mg/kg) significantly prolonged survival but did not completely eradicate the infection.

Fungizone at 2.0 mg/kg was acutely toxic and resulted in death of 50% of the treated mice. Of th se surviving treatment with 2.0 mg/kg Fungizone, all were cleared of the infection. All mice treated with 1.3 mg/kg Fungiz n were

completely cleared of the infection and tolerated multiple dosing at this level. Lower doses of Fungizone showed no overt toxicity, and prolonged survival but did not eradicate the infection.

In this model, AMB/cholesteryl sulfate dispersion was not as potent as Fungizone on a mg/kg basis; at the equivalent doses of 0.66 and 1.3 mg/kg, Fungizone reduced residual organ burdens of *C. immitis* more than AMB/cholesteryl sulfate dispersion did. The efficacy of Fungizone, however, was clearly limited by its toxicity at doses above 1.3 mg/kg while AMB/cholesteryl sulfate dispersion was tolerated at doses as high as 10 mg/kg.

B. *Cryptococcus neoformans*

The efficacy of AMB/cholesteryl sulfate dispersion was compared to Fungizone in female CD-1 mice infected with *Cryptococcus neoformans*. Mice were infected with *C. neoformans* and treated on days 4, 6, 8, 11, 13, and 15 post-infection with buffer controls, AMB/cholesteryl sulfate dispersion (0.8, 3.2, 6.4, 12.8, or 19.2 mg/kg), or Fungizone (0.2, 0.8, or 3.2 mg/kg). Mice were observed for toxicity for 49 days post-infection, at which time, surviving mice were killed and brain, lungs, liver, spleen, and kidneys and the burden of remaining infection determined.

Both AMB/cholesteryl sulfate dispersion and Fungizone were effective in treating murine systemic cryptococcoses with respect to prolongation of survival and reduction of organ burdens of *C. neoformans*. At 0.8 mg/kg, Fungizone and AMB/cholesteryl sulfate dispersion appeared to have a similar effect on both prolongation of survival and reduction of organ burdens. A dose of 3.2 mg/kg Fungizone was toxic, however, and r sulted in death of all treated mice. AMB/cholesteryl sulfate dispersion was effective in prolonging survival and reducing organ burdens at 3.2 and

Mice were immunosuppressed with Depo-Medrol and infected with *A. fumigatus*. More of the mice treated with 4.0 mg/kg of AMB/cholesteryl sulfate dispersion survived compared to the controls. No toxicity was seen in any of the AMB/cholesteryl sulfate dispersion-treated mice. Fungizone was toxic at 1.0 mg/kg, causing 30% of the mice to die the first day after treatment began.

Example 11

Inhibition of HIV Infectivity in Macrophage Cultures

This example shows the results of studies of treating HIV infected macrophage cultures with an AMB/cholesteryl sulfate colloidal dispersion (AMB/cholesteryl sulfate dispersion).

A. Isolation of Monocytes and Macrophage

HIV infected monocytes-macrophage were prepared essentially as described by Crowe et al. (29). Whole human peripheral blood was collected from healthy donors into tubes containing an anticoagulant. The whole blood sample was centrifuged (5000 rpm for 15 minutes) and the "buffy coat" cell layer isolated with a pipet. Peripheral blood mononuclear cells were isolated by centrifugation of the buffy-coat cell-layer over "FICOLL-HYPAQUE." The isolated cells were washed four time with serum free medium (RPMI 1640) to remove any contaminating platelets. The cells were then transferred to glass petri dishes containing "RPMI 1640" medium which was supplemented with 20% fetal calf serum. The plates containing the cells were incubated at 37°C for approximately one hour. The plates were then washed to remove non-adhering lymphocytes. Adherent monocytes-macrophages were r cover d from the plates by washing with 5μM EDTA in phosphate buffered salin (PBS) containing 5% fetal calf serum on ice for 10 minut s followed by scraping with a rubber spatula. The recovered

monocytes-macrophages were then harvested by centrifugation, resuspended in "RPMI 1640" medium with 10% HIV(-) complete human serum, and placed in "TEFLON" coated 48 well culture plates at approximately 2 X 10^6 cells/ml. Typically, cell viability had an initial decrease, over approximately a week, after which the cultures achieved a stable density which could be maintained for several months. Cell culture media were changed approximately every 7 days.

B. Isolation of HIV

A clinical monocytotrophic isolate of HIV designated HIV_{mn} was used in the following experiments. The isolate was obtained from a HIV positive donor and passaged in cell culture by Pan Data Systems of Rockville, Maryland (now known as Universal Biotechnology Technology, Inc.). Stock cultures of this isolate with an approximate titer of 1 X 10^5 $TCID_{50}$/ml were stored at -70°C until use. Reverse transcriptase activity as measured essentially by the method of Hoffman (16).

C. Infection of the Monocytes-Macrophage

Following adherence to the bottom surface of the multiwell culture plates, the human peripheral blood monocytes isolated as described above differentiated into macrophages with phagocytic activity and formed stable nearly confluent monolayers. Eight serial 5-fold dilution of the HIV_{mn} stock starting at 1:5 were prepared in growth medium containing 10 μg/ml polybrene. Macrophage plates were infected by the addition of 0.2 ml/well of the virus dilutions to each well (corresponding to multiplicities of infection of: 1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625. The virus was allowed to absorb for 90 minutes at 37°C after which tim the cell cultures were washed twice with serum-free medium to remove unabsorbed in culum.

D. Treatment of Infected Monocytes-Macrophage with AMB/cholesteryl sulfate dispersion and AZT.

AMB/cholesteryl sulfate dispersion was prepared as described in Example 9. Dilutions of reconstituted AMB/cholesteryl sulfate dispersion were made in sterile 5% dextrose to the following concentrations: 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 μg/ml. AZT (obtained from the Burroughs Welcome Company) was diluted in complete growth medium to concentrations of 10, 1, 0.1, and 0.01 μM. Immediately following washing of unbound virus inoculum from the cells, the medium containing the dilutions of AMB/cholesteryl sulfate dispersion or AZT were added to duplicate wells of the macrophages cultures, at each multiplicity of infection (a total of 16 wells for each drug concentration), and incubated for 10-14 days with the culture fluid being changed every 3-4 days with medium containing fresh drug at the proper dilution. Replication of HIV in control (untreated) cell cultures was followed daily. Once an acceptable control titer was achieved (i.e., $>10^4$ $TCID_{50}$/ml), the assay plates were lysed with Triton X-100 and assayed by a standard p24 antigen capture assay kit (17,18). Efficacy of the drug action was determined by comparing the concentrations of HIV p24 in treated versus control wells, at a given multiplicity of infection. The results for AZT and AMB/cholesteryl sulfate dispersion are presented in Figure 6 and 7, respectively.

In the case of AMB/cholesteryl sulfate dispersion (Figure 7), HIV replication is inhibited in approximately the same concentration range as AZT.

Although the invention has been described and illustrated with resp ct to specific embodiments, uses and methods f preparation, it will be appreciated that a variety f changes and modifications may be made without departing from the scope of the invention.

IT IS CLAIMED:

1. A method of inhibiting HIV infection in peripheral blood macrophage cells, as evidenced by an inhibition of HIV p24 antigen expression in the infected cells, comprising

exposing the infected cells to a composition containing particles of amphotericin B:cholesteryl sulfate, molar ratio 1:0.5 to 1:4, at a concentration of at least about 0.01 μM amphotericin B.

2. The method of claim 1, wherein the molar ratio of amphotericin B to cholesteryl sulfate is between about 1:1 and 1:2.

3. The method of claim 1, wherein the sizes of particles in the composition are between about 40-400 nm.

4. The method of claim 3, wherein the molar ratio of amphotericin B to cholesteryl sulfate is between about 1:1 and 1:2.

5. The method of claim 1, for use in treating a human subject infected with HIV, wherein the amount of said composition administered contains about 0.25-2.0 mg amphotericin B/kg human subject.

6. The method of claim 5, wherein said exposing is repeated at periodic intervals until there is produced a measurable improvement in at least one of the indications of HIV infection:

(a) a decrease in HIV antigen levels associated with HIV-infected cells;

(b) a decrease in HIV antigen levels in the bloodstream (antigenemia);

(c) a decrease in the titer of HIV particles in the bloodstream (viremia);

(c) a decrease in the level of reverse-transcriptase activity associated with HIV-infected cells.

(d) a decrease in the rate of HIV-induced destruction of CD4 positive T helper lymphocytes; or

(e) an increase in the absolute number of CD4 positive T helper lymphocytes in the peripheral circulation.

7. The method of claim 6, wherein said exposing includes

(a) producing an aqueous dispersion of amphotericin B and cholesteryl sulfate particles in a molar ratio of between 1:0.5 to 1:4, with particle sizes predominantly between 40-200 nm;

(b) lyophilizing the suspension in the presence of a cryoprotectant before the particle sizes in the suspension increase substantially; and

(c) reconstituting the lyophilized material with an aqueous medium to obtain a suspension of particles of sizes predominantly no larger than 400 nm.

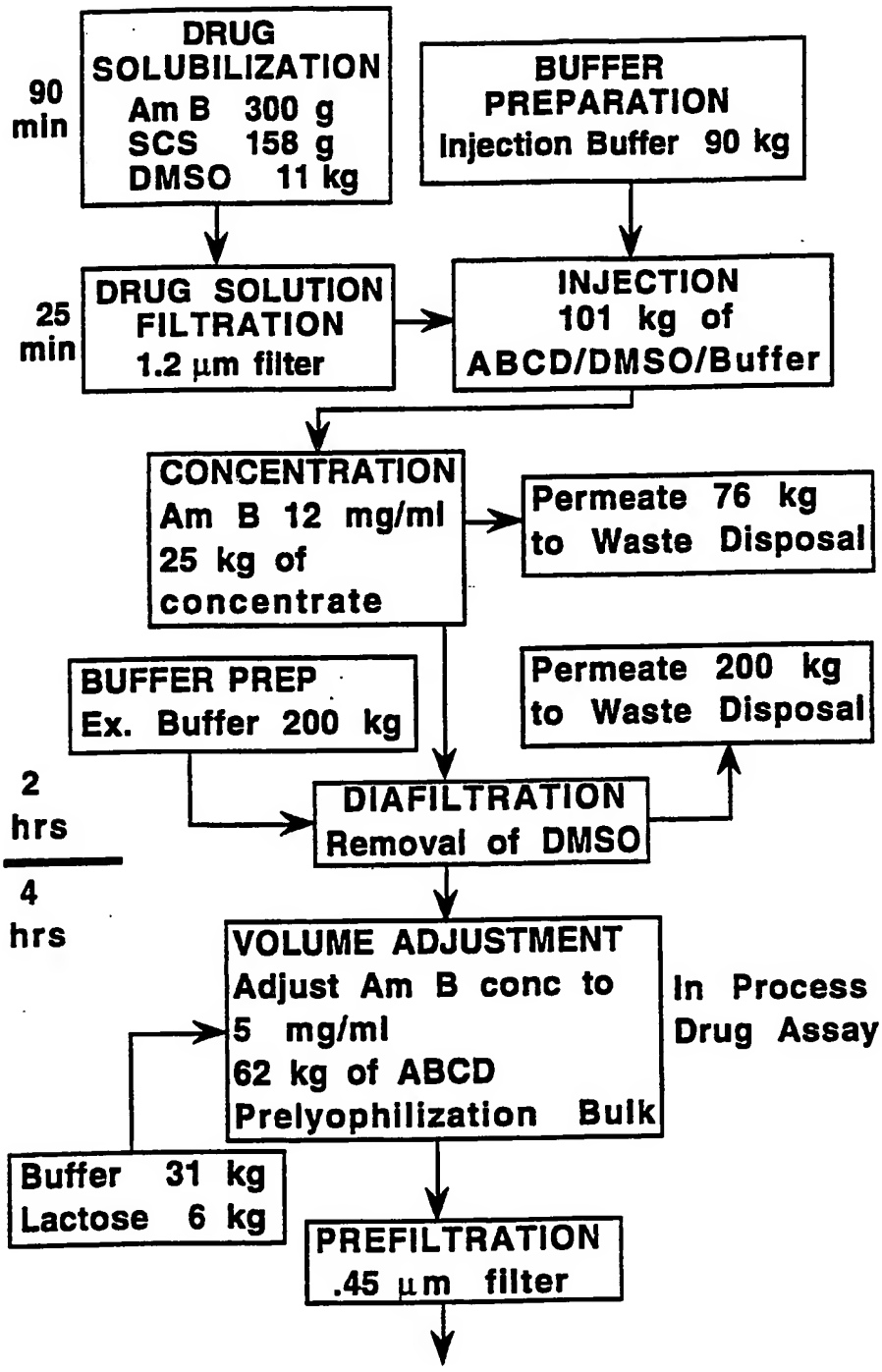
90 min
DRUG SOLUBILIZATION
Am B 300 g
SCS 158 g
DMSO 11 kg
BUFFER PREPARATION
Injection Buffer 90 kg
25 min
DRUG SOLUTION FILTRATION
1.2 µm filter
INJECTION
101 kg of
ABCD/DMSO/Buffer
CONCENTRATION
Am B 12 mg/ml
25 kg of
concentrate
Permeate 76 kg
to Waste Disposal
BUFFER PREP
Ex. Buffer 200 kg
Permeate 200 kg
to Waste Disposal
2 hrs
DIAFILTRATION
Removal of DMSO
4 hrs
VOLUME ADJUSTMENT
Adjust Am B conc to
5 mg/ml
62 kg of ABCD
Prelyophilization Bulk
In Process Drug Assay
Buffer 31 kg
Lactose 6 kg
PREFILTRATION
.45 µm filter

Fig. 1 (1/2)

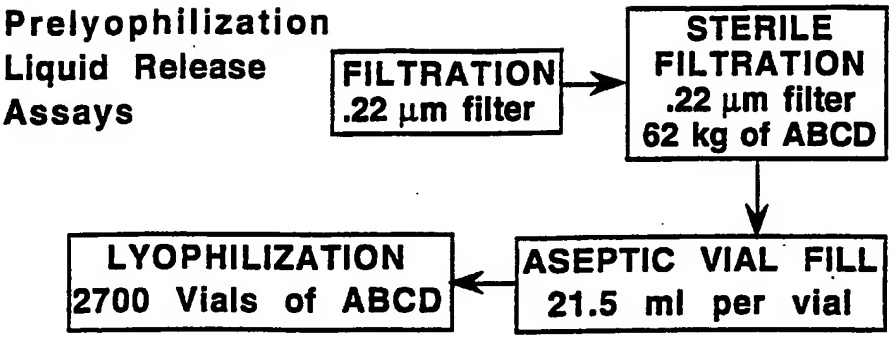
Prelyophilization
Liquid Release
Assays
FILTRATION
.22 µm filter
STERILE
FILTRATION
.22 µm filter
62 kg of ABCD
ASEPTIC VIAL FILL
21.5 ml per vial
LYOPHILIZATION
2700 Vials of ABCD

Fig. 1 (2/2)

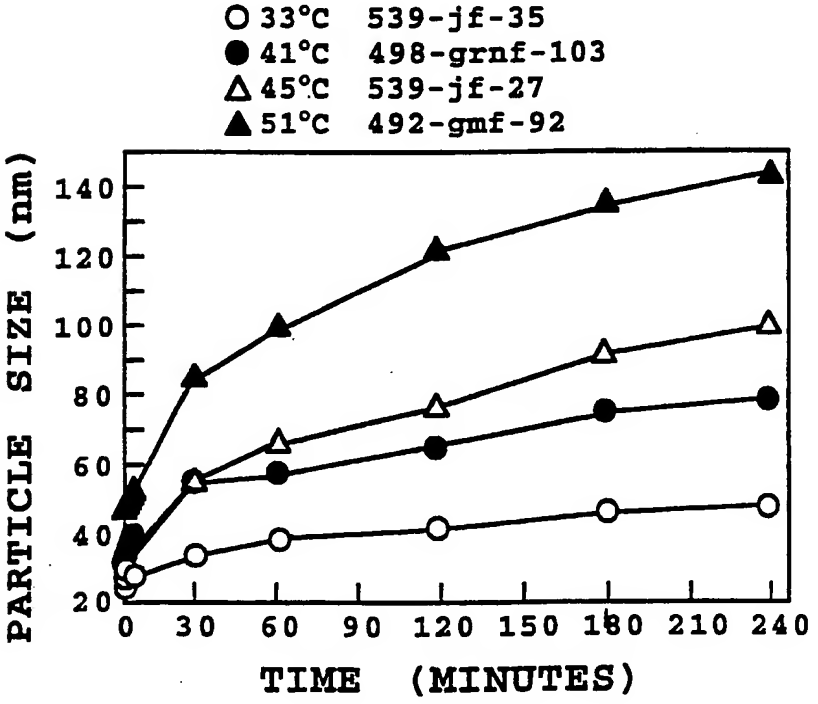
○ 33°C 539-jf-35
● 41°C 498-grnf-103
△ 45°C 539-jf-27
▲ 51°C 492-gmf-92
PARTICLE SIZE (nm)
20
40
60
80
100
120
140
0 30 60 90 120 150 180 210 240
TIME (MINUTES)

Fig. 2

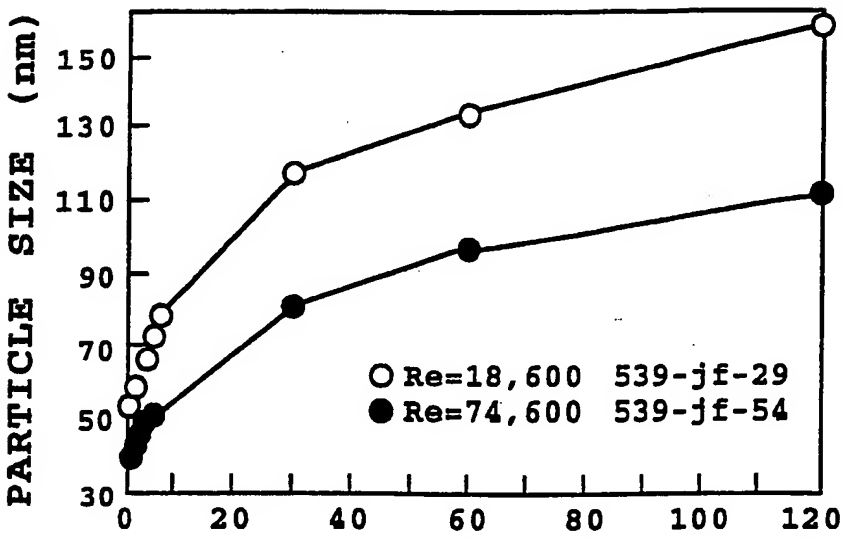
PARTICLE SIZE (nm)
150
130
110
90
70
50
30
0
20
40
60
80
100
120
Re=18,600 539-jf-29
Re=74,600 539-jf-54
TIME (MINUTES)

Fig. 3

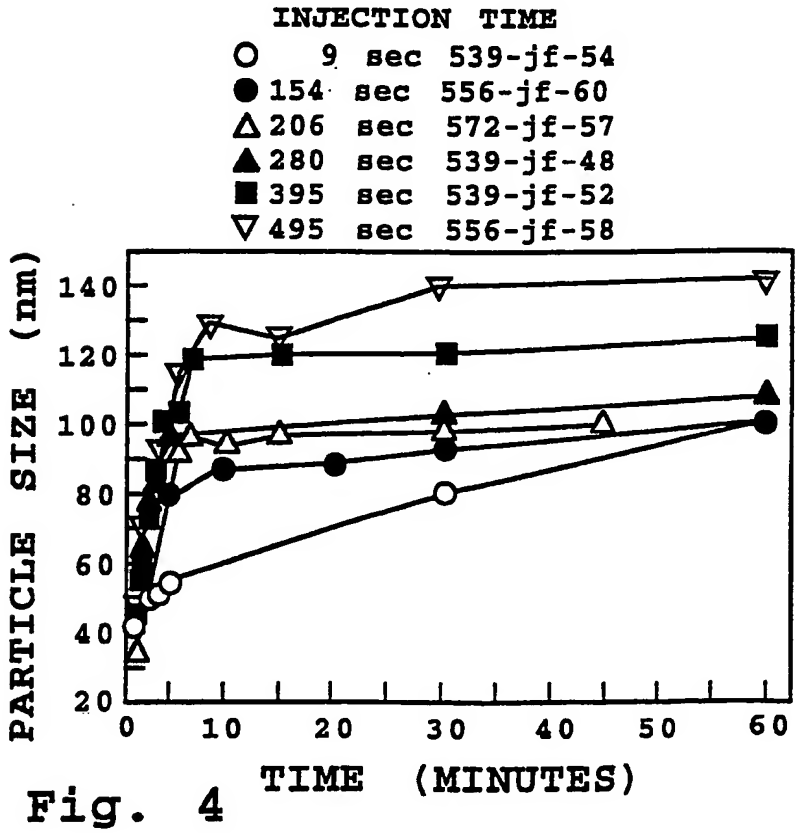
INJECTION TIME
9 sec 539-jf-54
154 sec 556-jf-60
206 sec 572-jf-57
280 sec 539-jf-48
395 sec 539-jf-52
495 sec 556-jf-58
PARTICLE SIZE (nm)
140
120
100
80
60
40
20
0
10
20
30
40
50
60
TIME (MINUTES)

Fig. 4

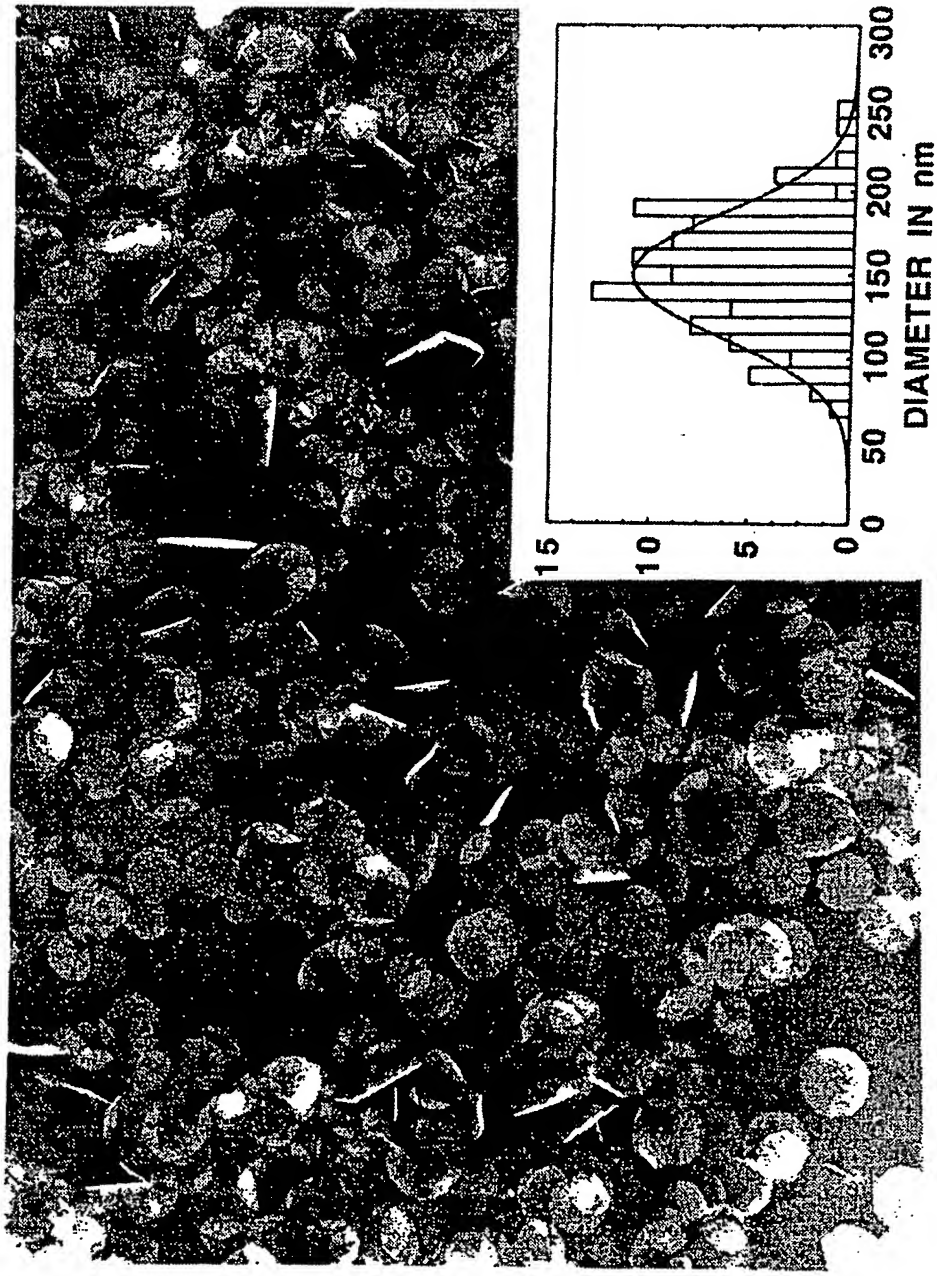

Fig. 5

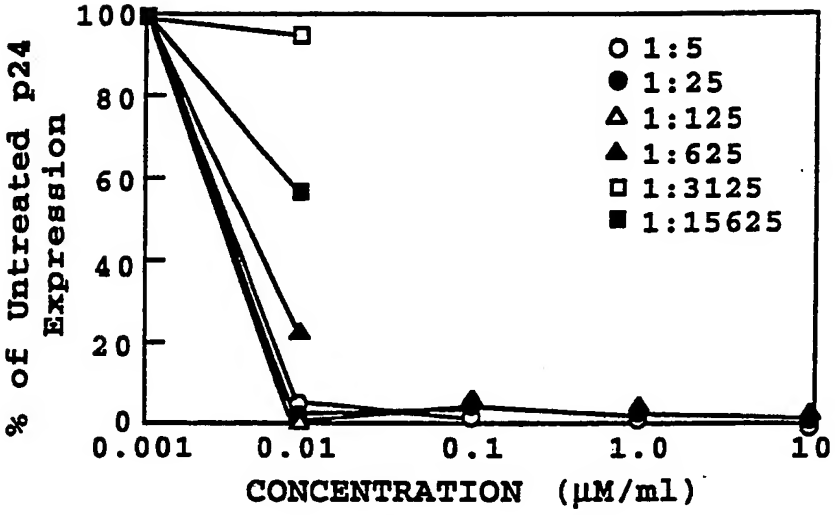
100
80
60
40
20
0
% of Untreated p24 Expression
0.001
0.01
0.1
1.0
10
CONCENTRATION (μM/ml)
1:5
1:25
1:125
1:625
1:3125
1:15625

Fig. 6

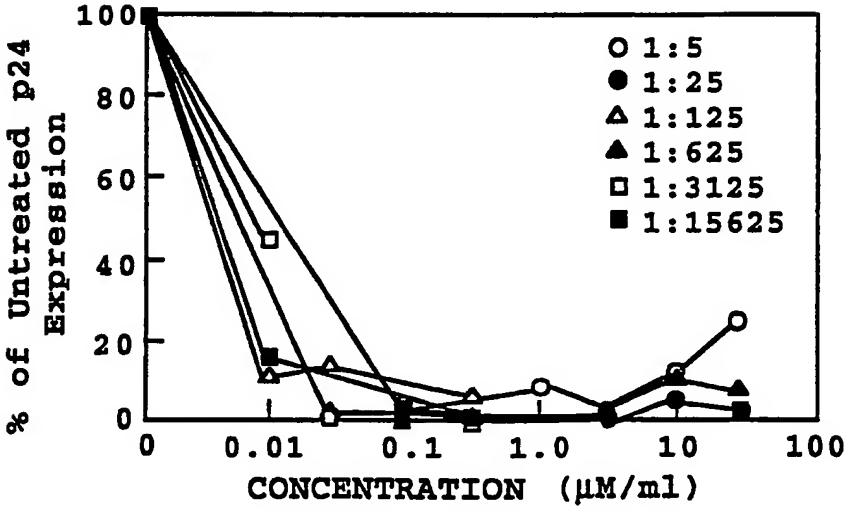
100
80
60
40
20
0
% of Untreated p24 Expression
0
0.01
0.1
1.0
10
100
CONCENTRATION (μM/ml)
1:5
1:25
1:125
1:625
1:3125
1:15625

Fig. 7

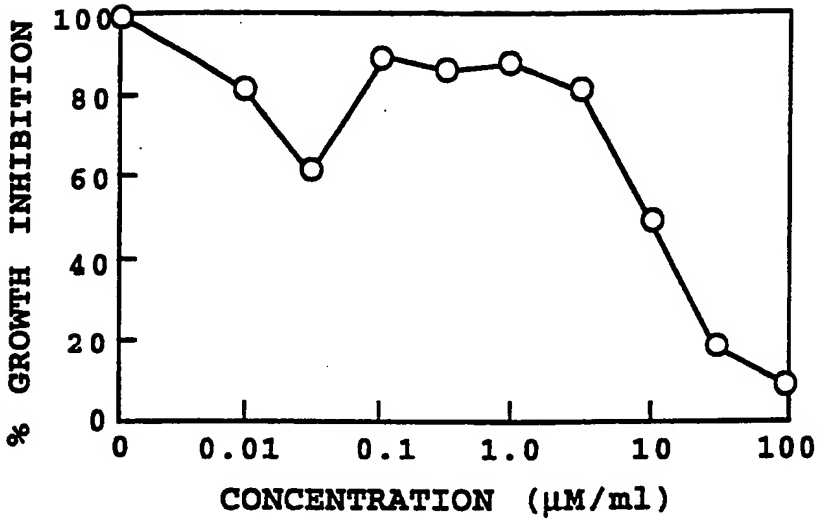
% GROWTH INHIBITION
100
80
60
40
20
0
0
0.01
0.1
1.0
10
100
CONCENTRATION (μM/ml)

Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/06788

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K31/71

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	WO,A,8 806 450 (LIPOSOME TECHNOLOGY, INC.) 7 September 1988 cited in the application see the whole document ---	1-7
Y	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES vol. 618, 28 February 1991, pages 586 - 588 L.S. GUO ET AL. 'PHARMACOKINETIC STUDY OF A NOVEL AMPHOTERICIN B COLLOIDAL DISPERSION WITH IMPROVED THERAPEUTIC INDEX' see the whole document ---	1-7

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 OCTOBER 1992	20. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	Dagmar Frank

Form PCT/ISA/210 (second sheet) (January 1985)

Mme Dagmar FRANK

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	THE JOURNAL OF INFECTIOUS DISEASES vol. 159, no. 4, 1989, pages 717 - 724 T.F. PATTERSON ET AL. 'TREATMENT OF EXPERIMENTAL INVASIVE ASPERGILLOSIS WITH NOVEL AMPHOTERICIN B/CHOLESTEROL-SULFATE COMPLEXES' see the whole document	1-7
Y	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY vol. 35, no. 6, June 1991, pages 1029 - 1034 S.W. SANDERS ET AL. 'SINGLE-DOSE PHARMACOKINETICS AND TOLERANCE OF A CHOLESTERYL SULFATE COMPLEX OF AMPHOTERICIN B ADMINISTERED TO HEALTHY VOLUNTEERS' see the whole document	1-7
Y	CLINICAL RESEARCH vol. 36, no. 1, 1988, page 41A O. PRAKASH ET AL. 'TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION WITH AMPHOTERICIN B' see the whole document	1-7
Y	WO,A,8 806 443 (THE LIPOSOME COMPANY, INC.) 7 September 1988 see abstract see page 5, line 7 - line 20; claims	1-7
Y	ANTIVIRAL RESEARCH vol. 11, no. 3, 1989, pages 119 - 126 D.R. PONTANI ET AL. 'INHIBITION OF HIV REPLICATION BY LIPOSOMAL ENCAPSULATED AMPHOTERICIN B' see the whole document	1-7
Y	US,A,5 032 404 (LOPEZ-BERESTEIN ET AL.) 16 July 1991 see the whole document	1-7
P,Y	INTERNATIONAL JOURNAL OF PHARMACEUTICS vol. 75, no. 1, 30 August 1991, pages 45 - 54 L.S. GUO ET AL. 'NOVEL ANTIFUNGAL DRUG DELIVERY: STABLE AMPHOTERICINB-CHOLESTERYL SULFATE DISCS' see the whole document	1-7

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III. DOCUMENTS CONSIDERED T BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	THIRTY-FIRST INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY vol. 31, October 1991, CHICAGO,USA page 147 DE WIT ET AL. 'SAFETY, TOLERANCE AND IMMUNOMODULATORY EFFECT OF AMPHOTERICIN B LIPID COMPLEX (ABLC) IN HIV INFECTED SUBJECT(S)' see abstract	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06788

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
ALTHOUGH CLAIMS 1-7 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/ANIMAL BODY, THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOUND/COMPOSITION.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9206788 SA 63716

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/10/92

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